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Chemokine Protocols

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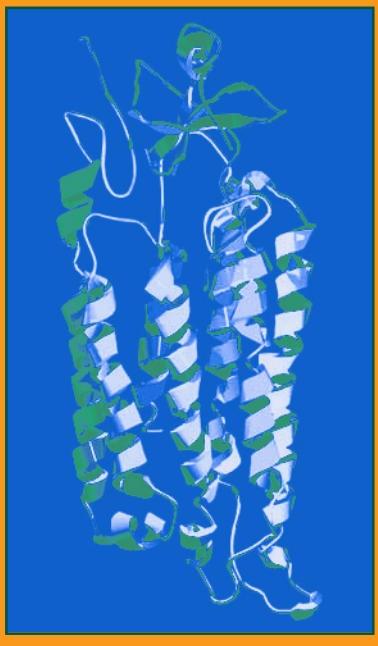

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Purification of Chemokines from Natural Sources

Jens-M. Schröder

1. Introduction

Chemokines (e.g., IL-8) were originally identified as chemotactic proteins obtained from different natural sources. These were purified by the use of a bioassay followed by N-terminal sequencing and then molecular cloning giving the full sequence and making it possible to express the chemokine as a recombinant protein. In this way we were sure that the biologically active chemokine is really released from natural sources. Today, using the genome walking strategy, we have discovered an ever-increasing number of novel genes encoding chemokines that were expressed in bacteria or eukaryotic cells and subsequently tested as the recombinant form for its biological activity.

Following reverse transcriptase-polymerase chain reaction (RT-PCR) investigations, Northern blot experiments, and *in situ* hybridization experiments much knowledge is obtained about localization of chemokine gene expression. Antibodies (often raised against chemokine peptide fragments or recombinant material) provide information about tissue localization of immunoreactive chemokines by immunostaining and presume the release of immunoreactive chemokine protein. Usually biological significance of the considered chemokine is extrapolated from these data. In principal, however, all these data are indirect. Thus, it is very important to provide direct proof of the release of biologically active chemokines from natural sources.

Purification of chemokines from natural sources requires special strategies. First of all, the detection system should be useful to detect the required chemokine at low concentrations, i.e., it is important to choose an assay system that allows the detection of low amounts of the chemokine in a screening system. Screening systems often used are Boyden chamber chemotaxis assay

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systems (*see* Chapter 11 and ***[1]***). Before starting the purification, the number of units of biological activity, which could be defined by estimating half maximum biological response doses (ED_{50}), should be determined. This is of particular importance, because with an estimated ED_{50} in chemotactic activity of most chemokines near 10–100 ng/mL, it is easy to calculate the approximate amount of a particular chemokine in biological samples. Due to the possible presence of a mixture of different chemokines and other chemotactic compounds, as well as inhibitors of migration, which make it impossible to estimate the units of activity, it is preferable to estimate the chemokine units after reverse phase high-performance liquid chromatography (HPLC) of the crude material.

Because chemokines are usually present in minute amounts, a number of points should be addressed prior to purification:

1. A large amount of biological material (cell culture supernatants or tissue) is required (whenever possible more than 0.5 L of culture supernatants).
2. The material needs to be concentrated to a low volume without significant losses of the chemokine.
3. Methods for microprotein purification have to be chosen, which should allow purification without large losses of material.
4. The solvents used for protein purification need to be compatible with the bioassay.
5. The right chromatographic strategy to purify the chemokines has to be chosen.

In the following sections methods for molecular characterization of chemokines from both cell culture supernatants and human tissue (lesional skin scales) will be described.

2. Materials

2.1. Bioassays

For bioassays freshly isolated blood cells need to be used.

1. Isolation of neutrophils polymorphonuclear (PMN) can be done using citrate/dextran/gelatine sedimentation (*see* **Note 1**). The following compositions are used:
   a. For isolation of PMN mix 100 mL freshly taken venous blood with 10 mL of a sterile acidic dextran containing anticoagulant solution (65 m*M* citric acid, 85 m*M* sodium citrate, and 20 g/L dextran T70 (Sigma, Munich, Germany). This solution can be stored at 7°C for four weeks. Gelatin solution (2.5% (w/v) in 0.9% aqueous saline [0.9% NaCl]) should be freshly prepared and stored at 37°C. Ammonium chloride for lysis of erythrocytes is used at 0.15 *M* in water, pH 7.0, and should be freshly prepared.

   b. Cells are washed and stored in phosphate buffered saline (PBS), pH 7.2, containing 128 m*M* NaCl, 8.1 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 2.7 m*M* KCl, and 0.1 % (w/v) bovine serum albumin (BSA, Fraction V, No. A 4503, Sigma) in the refrigerator (no longer than 4 h) until use.
2. Chemotaxis experiments (*see* **Note 2**): Use blind well Boyden chambers, that contain a volume of 100 μL in the lower compartment (Costar, Bodenheim, Germany). As filters use selfpunched (7 μm punch) polyvinylpyrrolidone containing polycarbonate filters (pore size 3 mm, Costar), which prior to use need to be washed with 1 *M* NaOH in 50 % (v/v) aqueous ethanol for 7 min followed by three washes in water (*see* **Note 3**). Use p-nitrophenyl-β-D-glucuronide (Sigma) at 10 m*M* in 0.1 *M* aqueous sodium acetate, pH 4.0, for β-glucuronidase detection (*see* **Note 4**).
3. Degranulation assays: For determining chemokines via induction of degranulation, PMN need to be preincubated with Cytochalasin B (Sigma, 5 mg/mL, from a frozen stock [1 mg/mL] in dimethylsulfoxide [DMSO]) for 5 min at 37°C (*see* **Note 5**). Determination of myeloperoxidase should be done using a solution of freshly prepared ophenylendiamindihydrochlorid (1 mg/mL, Sigma) in 0.1 *M* citrate/phosphate buffer, pH 5.0 (*see* **Note 6**).

2.2. Extraction of Chemokines from Biological Material

1. Suspend lesional tissue material in 0.1 *M* aqueous citric acid containing 50% (v/v) 96% ethanol (denaturated with heptane) (*see* **Note 7**).
2. Homogenizer (Ultraturrax®): use 2000 rpm for 60 min and chilling in an icewater mixture.
3. Ultrafiltration: YM2 filters, cutoff of 2 kDa (Amicon, Danvers, MA) are useful (*see* **Note 8**).

2.3 Chromatographic Separation of Chemokines

Use HPLC-quality solvents. All chromatographic steps are performed at room temperature (*see* **Note 9**).

1. Any HPLC or FPLC machine containing a pump, gradient mixer, UV detector, HPLC columns, and fraction collector can be used. UV detection should be done at 215 nm. Use solvents that do not show absorbance at 215 nm (i.e., acetonitrile, water, and trifluoroacetic acid [TFA]) (*see* **Note 10**). For MicroHPLC we use a Smart®HPLC system (Pharmacia).
2. For HPLC separation the following columns can be used:
   a. Heparin Sepharose cartridge (Hi Trap, 10 × 5 mm, 1 mL volume, Pharmacia).
   b. Preparative wide-pore (300 Å) reverse phase (RP-8) HPLC column (C8 Nucleosil with endcapping, 250 × 12.6 mm, 7 mm particle size, Macherey and Nagel, Düren, Germany) (*see* **Notes 11** and **19**).
   c. CN-propyl HPLC column (wide pore with endcapping, 5 mm, 250 × 4.6 mm; J.T. Baker, Gross Gerau, Germany).

d. Reversed phase (RP-18) HPLC column (narrow pore [125 nm], Nucleosil, 5 mm octadecylsilyl with endcapping [Bischoff, Leonberg, Germany].
e. TSK-CM-3SW cation exchange HPLC column (LKB, Bromma, Sweden, 125 × 12 mm) (*see* **Note 12**).
f. TSK-2000 size exclusion HPLC column (Pharmacia) (*see* **Note 13**).
g. Micro Mono S HPLC column for Smart®-System (Pharmacia).
h. Micro RP-18 (C2/C18) column for Smart®-System (Pharmacia).

2.4 SDS PAGE Analysis

1. For fast detection we use the Phast®-System (Pharmacia) with "high density gels" containing Tricine according to the manufacturer's instructions.
2. For high resolution, the method of Schägger and von Jagow *(2)* is used. In this case as sample buffer 50 m*M* Tris-HCl, 4% (w/v) SDS, 12% (w/v) glycerol, pH 6.8, containing 8*M* urea, is used.
3. Gels with the dimension 130 × 100 × 1 mm are used and electrophoresis is done in the presence of 8 *M* urea for 18 h at 10 mA current, 30 V power (power limit 10W) at room temperature (*see* **Note 14**).
3. Fixation of chemokines is done for 30 min with aqueous 2-propanol (30% [v/v]) containing 10% (v/v) acetic acid and 0.3% (v/v) glutaraldehyde (*see* **Note 15**).
4. Proteins are stained with 0.03% (w/v) silver nitrate in deionized water followed by developing with a solution of 10% saturated aqueous Na_2CO_3 solution containing 0.1% (v/v) of saturated aqueous formaldehyde (40% [v/v]). Development is terminated by acetic acid (3% [w/v] in water) (*see* **Note 16**).

3. Methods

3.1. Chemokine Purification

3.1.1. Chemokine Purification from Cell Culture Supernatants

1. Acidify cell culture supernatants with formic acid to pH 3.0.
2. Concentrate the supernatant on a ultrafiltration membrane to 35 mL.
3. Take out the concentrate, bring it to pH 8.0, and centrifuge (*see* **Note 17**).
4. Take the supernatant and apply it to a heparin column.
5. Wash the column with 3 vol of equilibration buffer: 10 m*M* Tris-citrate, pH 8.0.
6. Strip the bound material from the column by the use of 3 mL glycine buffer, pH 2.0.
7. Diafiltrate against 0.1% trifluoroacetic acid.
8. Apply the stripped material to a preparative RP-8 HPLC column using a 5–10 mL loop (*see* **Note 18**).
9. Separate proteins by elution with a gradient of increasing concentrations of acetonitrile. Turn the detector on 215 nm and choose the integrator attenuation appropriate to protein amounts you expect (*see* **Note 19**).
10. Separate peaks manually according to the appearance of UV-absorbing peaks and shoulders (*see* **Note 20**).

11. Place fractions immediately in a refrigerator.
12. Take off an aliquot of each fraction for bioassays, solid phase ELISA, or SDS-PAGE analysis using a microtiter plate.
13. Lyophilize fractions chosen for further purification.
14. Dissolve the residue in 100 mL equilibration buffer for micro-Mono S HPLC.
15. Apply the sample to a micro-Mono S HPLC column and elute proteins with a salt gradient. Turn the UVdetector on 215 nm. Separate peaks manually according to absorbance at 215 nm.
16. Take out an aliquot of each fraction for bioassay, immunoassay, or SDS-PAGE analysis (*see* **Note 21**).
17. Apply fractions chosen for further purification onto a microreversed-phase HPLC column (C2/C18) and elute proteins with increasing concentrations of acetonitrile. Turn the UV-detector on 215 nm and separate peaks manually.
18. Take off an aliquot of each fraction for testing.
19. Store fractions below –70°C until further use.

3.1.2. Chemokine Purification from Tissue

1. Suspend lesional tissue (scales, skin, polyps, etc.) in acidic aqueous ethanol and homogenize.
2. Centrifuge and use supernatant.
3. Concentrate the supernatant to 25 mL, adjust to pH 8.0, and then freeze it (below –30°C) until further use.
4. For further purification, thaw the sample, centrifuge it, and apply it to a heparin column.
5. Continue purification as described in **Subheading 3.1.1., step 5**.

3.1.3. Bioassays for Detection of Chemokines in HPLC Fractions

3.1.3.1. Chemotaxis

1. Isolate white blood cells from freshly taken blood.
2. Fill the lower part of the Boyden chamber with appropriately treated HPLC fractions (*see* **Note 22**).
3. Cover the lower part of the chamber with the chemotaxis filter (*see* **Note 23**).
4. Screw the upper part of the Boyden chamber tight to the lower part.
5. Suck away carefully fluid remaining in the upper part (*see* **Note 24**).
6. Add 100 mL cell suspension to the upper part.
7. Cover the chamber with a moistened slide.
8. Incubate the chamber for 1 h when neutrophils are used (or 2 h, when eosinophils are used).
9. Suck away the fluid and remaining cells present in the upper part of the chamber, open the chamber and remove the filter carefully.
10. Add to each chamber 10 μL Triton X100 solution, incubate 5 min and then transfer the whole volume to a microtiter plate (*see* **Note 25**).

11. Lyse defined numbers of cells, determine the β-glucuronidase content, and establish a calibration curve (*see* **Note 26**).
12. Add enzyme substrate and substrate buffer and incubate over night.
13. Add stopping buffer to terminate enzymatic reaction and determine product formation.

3.1.3.2. Enzyme Release

1. Fill HPLC fractions into a microtiter plate (each 10–30 μL/well), add 10 μL PBS containing BSA, freeze, and then lyophilize for 30 min. Then add 100 μL PBS containing Ca^{++}, Mg^{++} and BSA and warm up to 37°C for 10 min.
2. Treat prewarmed neutrophils with Cytochalasin B.
3. Add pretreated PMN in 100 μL PBS to each well and incubate for 30 min at 37°C.
4. Centrifuge the plate.
5. Take out carefully 100 μL supernatant from each well.
6. Add peroxidase substrate dissolved in acidic citrate/phosphate buffer and incubate 20 min (maximum) in the dark.
7. Stop the enzymatic reaction by adding 2 *M* H_2SO_4 and determine absorbance at 486 nm using a microtiter plate photometer.

3.1.4. Solid Phase ELISA for Chemokine Detection

1. Add aliquots of HPLC fractions to ELISA plates and lyophilize the plate (30 min). Then add coating buffer to each well and incubate overnight in the refrigerator.
2. Take off the fluid and incubate with blocking buffer for 30 min.
3. Wash the plate, add chemokine antibody solution, and incubate for 1 h.
4. Take off the fluid, wash the plate, and add enzyme linked secondary antibody.
5. Wash again, incubate with enzyme substrate, and determine absorbance in a microtiter plate reader (*see* **Note 27**).

3.1.5. Gel Electrophoresis of Chemokines

3.1.5.1. Tricine Electrophoresis in the Presence of Urea

1. Mix fractions to be tested with 10 μL sample buffer and boil for 10 min. Then load sample on the stacking gel and separate electrophoretically in the presence of 8 *M* urea.
2. Fix chemokines in the gel with fixation solution.
3. Stain proteins with silver nitrate solution (*see* **Note 28**).

3.1.5.2. Gel Electrophoresis using Commercially Available Gels

1. Treat samples as in **Subheading 3.1.5.1.**, however using only 1 μL sample buffer (*see* **Note 29**).
2. Use commercially available high density gels and follow the instructions for performing electrophoresis, in **Subheading 2.4.**

3. Fix proteins with fixation solution.
4. Stain proteins with silver nitrate in deionized water.

4. Notes

1. Ficoll® is also useful for PMN isolation instead of citrate/dextrane giving similar results in PMN chemotaxis and degranulation ***(1)***. Percoll® treatment in our hands resulted in lower efficacy of PMN chemotaxis. When PMN preparations are used, be aware that they may contain contaminating eosinophils, leading wrongly to "neutrophil chemotactic" chemokines! Eosinophils can be separated by Percoll centrifugation ***(1)***.
2. For detection of neutrophil or eosinophilchemotactic chemokines, we have used this method for more than 10 years and have been successful in detecting a number of chemokines ***(3–7)*** as well as novel chemotactic lipids ***(8,9)***. This method does not seem to be useful for detection of monocyte and lymphocyte attractants (*see* Chapter 11).
3. Commercially available, polyvinylpyrrolidone (PVP)-free chemotaxis filters are not useful in this system, because leukocytes stick to the lower surface. In the case of eosinophils, 3 μm filters can also be used.
4. p-Nitrophenyl-β-D-glucuronide is much cheaper than phenolphthalein-β-D-glucuronide which is usually used.
5. Pretreatment of PMN with Cytochalasin B is essential for release of enzymes after stimulation with chemokines ***(1)***. Without Cytochalasin B, no enzyme release is detectable.
6. Instead of myeloperoxidase as a marker enzyme, β-glucuronidase, or elastase can be used with similar success. Myeloperoxidase measurement gives the fastest results, which might be important for doing additional HPLC the same day.
7. Instead of ethanol, acetonitrile can also be used. In the absence of organic solvents, we have had big problems with the extracts giving turbid solutions after centrifugation with a high content of fines (lipid drops mixed with solid particles). Ignoring this phenomenon has usually resulted in HPLC problems, such as high pressure error and giving "memory compounds" on the HPLC column.
8. The recovery of chemokines using Amicon (Danvess, MA) filters was found to be highest when acidic solutions containing a small percentage (20–30%) of water-soluble organic solvents were used. Use the right diameter of the filter to avoid losses due to unappropriated ratio of filter diameter and (final) sample volume.
9. It is our experience that all chemokines we have isolated are remarkably heat stable as well as protease resistant. Losses of material seen in some cases usually come from sticking to the surfaces rather than degradation. Once chemokines stick to surfaces (glass, siliconized glass, plastic), we were unable to reverse the process by washing with organic solvents. Therefore, our strategy to avoid sticking is to add organic solvents whenever possible at acidic pH to chemokine-containing solutions.
10. UV detection at 215 nm allows quantitation of protein content and thus

estimation of the chemokine amounts in purified peaks. We used ubiquitin for calibration (Sigma).

11. A wide variety of reverse-phase columns obtained from different manufacturers can be used. We found that the best results were obtained with columns that have already been used for some time (6 mo) for lipid analyses. These columns revealed separation properties different to those of new columns.
12. Be aware that cation-exchange HPLC should be performed at slightly acidic pH in the presence of 20–30% acetonitrile, as was used for isolation of natural eotaxin ***(7)***.Use cation exchange columns as early as possible in the purification protocol. It is our experience that cation exchange chromatography reveals dramatic losses of chemokines when used as one of the last purification steps!
13. The silica-based TSK-2000 size exclusion HPLC column has excellent separation properties for proteins in MW range 2000–20,000, when 0.1% aqueous TFA is used as eluent ***(3,10)***. We have not seen this property when solvents at neutral pH were used or with other size-exclusion HPLC columns (resin-based as well as zirkoniumoxide-based). The use of 0.1% aqueous TFA solution also dramatically increases the recovery of chemokines. Nevertheless, the TSK-2000 column should be used only when other methods of purification cannot be applied.
14. With this method, we obtained highest resolution of bands. We were able to separate the different 77, 72, and 69 residues containing forms of IL-8 ***(1,11)***.
15. Fixation is a big problem for chemokines. The use of glutaraldehyde is compelling for detection of low amounts (<10 ng per lane) of chemokines.
16. When highly sensitive detection is necessary, the gels can be destained with $K_3[Fe(CN)_6]$ until background is cleared. Bands should never be completely destained. After washing out the destaining solution, staining can be repeated as described. The band intensity now does not reflect the relative amounts of the applied proteins.
17. In our hands, directly concentrating cell supernatants at pH 8.0 revealed lower recoveries than concentrating at low pH.
18. The volume of the applied material does not affect resolution, when it contains organic solvents at concentrations lower than 20%. In the case of higher content, samples should be lyophilized first and then should be dissolved in 0.1% aqueous TFA.
19. We also have tested 2-propanol instead of the very toxic acetonitrile. Resolution was far less than that seen with acetonitrile. 2-propanol can be used for HPLC with the CN-propyl HPLC column (*see* **Subheading 2.3., item 1**).
20. We are separating peaks by hand when bioassays have to be performed. It is our experience that it is easier to combine fractions than to rechromatograph wrongly mixed fractions.
21. Do not forget to add 10 μL BSA containing PBS to each fraction/aliquot present in the microtiter plate prior to lyophilization when a bioassay will be performed.
22. Up to 1% of the fraction volume in the assay system will be tolerated without affecting chemotaxis. When higher percentages need to be used these should be lyophilized in the presence of BSAcontaining PBS prior to bioassay.

23. Pretreatment of polycarbonate filters with ethanolic NaOH enhances responsiveness towards IL-8, possibly by expression of anionic carboxylate ions. Because NaOH dissolves polycarbonates, and therefore lowers filter thickness and enhances pore size, do not extend incubation time and concentration of NaOH!
24. The remaining fluid contains chemoattractant. If this is not sucked away, cells are exposed to attractant prior to chemotaxis, which results in desensitization and thus lower chemotactic responses. This is one of the major critical points affecting standard deviation. Be aware from sucking chemoattractant from the lower chamber (through the filter), which results in air bubbles and inappropriate migration. Be aware that all vials used for buffer preparations used for chemotaxis should be free of detergents that are used for washing. We found that traces of detergents dramatically decreased the motility of leukocytes in the chemotaxis assay system.
25. Triton X100 lyses cells in the lower compartment including those sticking to the lower chamber. It is better to add detergent to the chamber than to transfer migrated cells to a plate containing Triton X100.
26. The calibration curve of eosinophils is more steep than that of neutrophils ***(1)***. Therefore lower numbers ($<10^5$ cells) per chamber give good results with the less abundant eosinophils. Contamination of eosinophil preparations by neutrophils (<30%) is usually not a problem due to the more steep calibration curve. Use IL-8 as a control in the case of higher neutrophil contamination. If this gives strong peaks of activity neutrophil content might be too high to detect solely eosinophil chemotactic chemokines.
27. In the case of too high protein concentrations or high amounts of strongly cationic proteins, solid-phase ELISA will give low extinctions or false negatively no results! Either use less volume of fraction, which allows *all* proteins present to stick to the surface of the ELISA plate, or use sandwich ELISA.
28. Coomassie staining is only useful when high concentrations of chemokines are available (>1 μg per band). We have never used Coomassie staining for chemokines, because silver staining allows detection of less than 10 ng chemokine and therefore saves material.
29. For practical reasons, we have neither reduced samples nor boiled them, and have not had any problems in the analyses of chemokines. We have found that avoiding boiling reduces losses.

References

1. Schröder, J. M. (1997) Identification and structural characterization of chemokines in lesional skin material of patients with inflammatory skin disease. *Methods Enzymol.* **288,** 26,629.
2. Schägger, H. and von Jagow, G. (1987) Tricinesodium dodecyl sulfatepolyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDA. *Anal. Biochem.* **166,** 368–379.
3. Schröder, J. M., Mrowietz, U., Morita, E., and Christophers, E. (1987) Purification and partial biochemical characterization of a human monocytederived,

neutrophil activating peptide that lacks interleukin 1 activity. *J. Immunol.* **139,** 3474–3483.
4. Schröder, J. M., Persoon, N. L. M., and Christophers, E. (1990) Lipopolysaccharidestimulated human monocytes secrete, apart from neutrophilactivating peptide 1/Interleukin 8, a second neutrophil activating protein. *J. Exp. Med.* **171,** 1091–1100.
5. Kameyoshi, Y., Dörschner, A., Mallet, A. I., Christophers, E., and Schröder, J. M. (1992) Cytokine RANTES released by thrombinstimulated platelets in a potent attractant for human eosinophils. *J. Exp. Med.* **176,** 587–592.
6. Noso, N., Sticherling, M., Bartels, J., Mallet, A. I., Christophers, E., and Schröder, J. M. (1996) Identification of an N-terminally truncated form of the chemokine RANTES and granulocyte-macrophage colony-stimulating factor as major eosinophil attractants released by cytokine-stimulate dermal fibroblasts. *J. Immunol.* **156,** 1946–1953.
7. Mochizuki, M., Bartels, J., Mallet, A. I., Christophers, E., and Schröder, J. M. (1998) IL4 induces eotaxin: a possible mechanism of selective eosinophil recruitment in helminth infection and atopy. *J. Immunol.* **160,** 60–68.
8. Schwenk, U., Morita, E., Engel, R., and Schröder, J. M. (1992) Identification of 5oxo15hydroxy6,8,11,13eicosatetraenoic acid as a novel and potent human eosinophil chemotactic eicosanoid. *J. Biol. Chem.* **267,** 12,482–12,488.
9. Kahlke, B., Brasch, J., Christophers, E., and Schröder, J. M. (1996) Dermatophytes contain a novel lipidlike leukocyte activator. *J. Invest. Dermatol.* **107,** 108–112.
10. Irvine, G. B. and Shaw, C. (1986) Highperformance gel permeation chromatography of proteins and peptides on columns of TSKG2000SW and TSKG3000SW: a volatile solvent giving separation based on charge and size of polypeptides. *Anal. Biochem.* **155,** 141–148.
11. Schröder, J. M., Gregory, H., Young, J., and Christophers, E. (1992) Neutrophilactivating proteins in psoriasis. *J. Invest. Dermatol.* **98,** 241–247.

2

Cloning of Novel Chemokines Using a Signal Sequence Trap Method

Toshio Imai

1. Introduction

Precursors of most secreted and cell surface molecules carry signal sequences at their amino termini. The method coined as signal sequence trap ***(1–3)*** takes advantage of the presence of N-terminal signal sequences in most precursor forms of secretory proteins and transmembrane proteins, which are necessary for the proper orientation of the N-terminal of mature forms inside endoplasmic reticulum and exocytotic vesicles. This method enables to selectively clone cDNA species encoding intercellular signal-transducing molecules without biologic assays. In this chapter, an efficient signal sequence trap method based on an Epstein-Barr virus shuttle vector is described ***(2)***.

5' portion-enriched cDNAs are synthesized using the 5' rapid amplification of *c*DNA ends (RACE) technique ***(4)*** as follows. First-strand cDNAs are synthesized from poly(A)+ RNA by using random hexanucleotide primers and reverse transcriptase. After mRNA templates are destroyed by alkali-treatment, an anchor oligo(dC) sequence is added to the 3' ends of cDNAs by using terminal deoxynucleotidyl transferase and dCTP. The second-strand DNA is synthesized by priming with an anchor primer which contains a *Sal*I site and an oligo(dG) tail. The double-stranded DNAs are sonicated to prepare short fragments. After blunting with T4 DNA polymerase, adapters are ligated to the fragments. DNA fragments are separated by agarose gel electrophoresis, and fragments consisting of 300–600 bp are recovered. The fragments that contained 5' noncoding regions and partial coding regions are then amplified by PCR using primers complementary to a portion of the anchor primer and the ligated adapter. Amplified DNA fragments are digested with *Sal*I and *Xba*I, separated by gel electrophoresis, and recovered from agarose gels.

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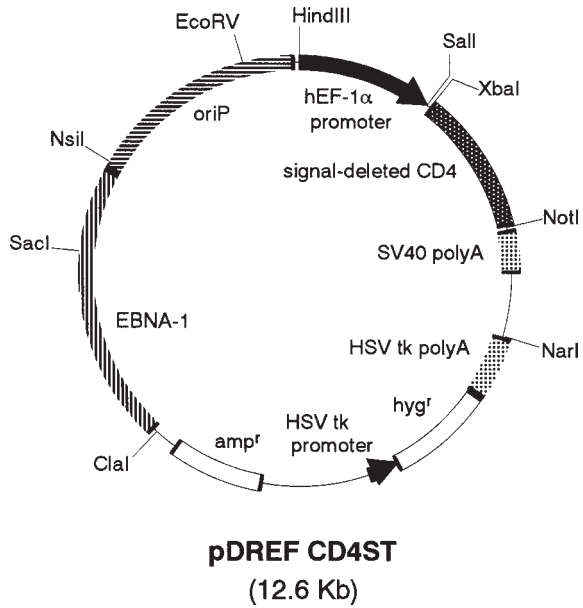

Fig. 1. Schematic diagram of the signal sequence trap vector pDREF-CD4ST. pDREF-CD4ST contains the EF-1α promoter, signal sequence-deleted CD4, the hygromycin resistant gene for selection (hygr), the EBNA-1 gene, and the EBV origin for episomal replication (oriP). 5' terminal-enriched cDNAs are inserted between *Sal*I and *Xba*I sites and expressed as fusion proteins with signal sequence-deleted CD4.

An expression library is constructed by inserting 5' portion-enriched cDNAs into the *Sal*I and *Xba*I sites upstream of signal sequence-deleted CD4 cDNA in an Epstein-Barr virus trapping vector, pDREF-CD4ST (**Fig. 1**) ***(2)***. This plasmid vector allows highly efficient transformation of human lymphoblastoid Raji cells with full representation of an expression library. Exogenous signal sequences allow CD4 fusion proteins to be expressed on the cell surface if cloned in frame. The advantage of this stable expression system is that cells positive for the reporter protein (CD4) can be enriched repeatedly by cell sorting. Furthermore, Epstein-Barr virus shuttle vector that is maintained as episomes can be readily isolated from transformants ***(5,6)***.

After electroporation into Raji cells, CD4 antigen-positive cells are enriched by repeated cell sorting and plasmids are recovered in *Escherichia coli*. Individual plasmids are retransfected and clones that induce expression of CD4 antigen on the cell surface were identified. Positive clones are sequenced and their deduced amino acid sequences are examined for the presence of a signal peptide. If a stretch of hydrophobic amino acids is detected immediately

following the initiator methionine ***(7)***, their putative protein products are analyzed by using computer program designated to search for signal peptides ***(8)***.

2. Materials

2.1. Synthesis of 5' Portion-Enriched cDNA

1. For first-strand cDNA synthesis: SUPERSCRIPT II RT reverse transcriptase (200 U/μL) supplemented with 5X RT buffer (250 m*M* Tris-HCl, pH 8.3, 375 m*M* KCl, 15m*M* $MgCl_2$) (Gibco-BRL), random hexamers (50 ng/μL), 0.1 *M* DTT, 10 m*M* dNTP mix, and DEPC-treated water. These solutions should be prepared as RNase-free. We utilize solutions supplemented with cDNA synthesis kit (Gibco-BRL)
2. For mRNA degradation; 6 *N* NaOH, 6 *N* acetic acid
3. Terminal deoxinucleotidyl transferase (TdT) (15 U/μL) supplemented with 5X TdT buffer (200 m*M* potassium cacodylate, pH 7.2, 10 m*M* $CoCl_2$, 1 m*M* DTT) (Gibco-BRL).
4. For second-strand DNA synthesis: Oligo-dG anchor primer (5'-CTACTA CTACTAGGCCACGCGTCGACTAGTAC(G)16-3'), *E. coli*. DNA polymerase I (10 U/μL) (Gibco-BRL), 10X Pol I buffer (100 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, 100 m*M* DTT), and T4 DNA polymerase (10 U/μL) (Gibco-BRL).
5. For Adapter ligation: T4 DNA ligase (1 U/μL) supplemented with T4 DNA ligase buffer (Gibco-BRL), Uni-Amp Adaptor, *Xba*I (Clontech, Palo Alto, CA).
6. For purification of cDNA fragments from agarose gels: PCR Prep Kit (Promega).
7. For PCR: Taq DNA polymerase I (5 U/μL) supplemented with 10X PCR buffer (200 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl) and 25 mM $MgCl_2$ (Takara), UAP1 Primer (5'-CCTCTGAAGGTTCCAGAATCGATAG-3'), UAP2 primer (5'-CTA CTACTACTAGGCCACGCGTCGACTAGTAC-3')
8. For restriction enzyme digestion: 10X T buffer (T4 DNA polymerase buffer): 330 m*M* Tris-acetate, pH 7.9, 660 m*M* K-acetate, 100 m*M* Mg-acetate, 5 m*M* DTT and 200 m*M* spermidine (Sigma). Restriction enzymes; *Sal*I and *Xba*I (Takara).
9. Other solutions: TE (10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA); Phenol/CIAA saturated with TE; 2 m*M* dCTP; 7.5 *M* NH_4OAc; EtOH; 70% EtOH, and sterile water.

2.2. Construction Signal Sequence Trap Library

1. Signal sequence trapping vector: pDREF-CD4ST vector contains EF-1 alpha promotor ***(9)***, signal sequence-deleted CD4 gene, hygromycin resistance gene, EBV oriP, EBNA-1 gene, and ampicillin resistance gene. This vector can be distributed upon request.
2. For preparation of vector fragment: Chromaspin TE-1000 (Clontech).
3. For electroporation: ELECTROMAX DH10B competent cells with pUC119 control plasmid (Gibco-BRL). We use Gene Pulser (Bio-Rad, Richmond, CA) equipped with Pulse Controller and *E. coli* Pulser cuvet with 0.1-cm gap (Bio-Rad) for electroporation of *E. coli*.

4. Primers used for analysis of insert DNA: EF seq F primer (5'-CCTCAGA CAGTGGTTCAAAG-3' and CD4 ST seq R primer (5'-TGTACAGGTCA GTTCCACTG-3')
5. Other solutions: yeast tRNA (1 μg/μL), S.O.C. medium (Gibco-BRL), LB medium, 1.2% agar-LB plate containing 100 μg/mL ampicillin.

2.3. Signal Sequence Trap

1. Monoclonal antibody (mAb) to human CD4: OKT4 (ATCC) and FITC-Leu3a (Becton Dickinson).
2. Sheep antimouse IgG-coated magnetic beads (Dynabeads M450, Dynal #11001).
3. Magnetic separator (Advanced Magnetics, Cambridge, MA).

3. Methods

3.1. Synthesis of 5′ Portion-Enriched cDNA

3.1.1. First-Strand cDNA Synthesis

1. Add 3 μL (150 ng) of random hexamers to a sterile 1.5-mL microcentrifuge tube. Add 5 μg of poly(A)$^+$ RNA, and DEPC-treated water to a total vol 10 μL. Heat the mixture to 70°C for 10 min, and quick-chill on ice. Spin down the reaction mixture, and add the following: 4 μL 5X RT buffer; 2 μL 0.1 *M* DTT; 1 μL 10 m*M* dNTP mix.
2. Incubate at 37°C for 2 min. Add 3 μL of SUPERSCRIPT II (200 U/μL). Incubate at 37°C for 1 h, then place on ice. Spin down the reaction mixture.

3.1.2. RNA Hydrolysis

1. Add 1.5 μL of 6 *N* NaOH to the 20 μL of cDNA and incubate at 70°C for 20 min to hydryze mRNA. Add 1.5 μL of 6 *N* acetic acid and 127 μL of TE.
2. Add 150 μL of Phenol/CIAA, vortex thoroughly, and centrifuge at room temperature for 5 min at 10,000*g*. Carefully remove 140 μL of the upper aqueous layer, and transfer it to a new 1.5-mL microcentrifuge tube.
3. Add 70 μL of 7.5 *M* NH4OAc, followed by 500 μL of absolute EtOH (–20°C). Incubate at –20°C for 30 min. Centrifuge at room temperature for 10 min at 10,000*g*.
4. Remove the supernatant, and overlay the pellet with 0.5 mL of 70% EtOH (–20°C). Centrifuge at room temperature for 2 min at 10,000*g*, and remove the supernatant.
5. Dry the cDNA at 37°C for 10 min to evaporate residual ethanol, and dissolve the cDNA in 16.5 μL of sterile H_2O.

3.1.3. TdT Tailing of cDNA

1. Add the following to a new 0.5-mL microcentrifuge tube: 16.5 μL cDNA sample; 5.0 μL 5X TdT buffer; 2.5 μL 2 m*M* dCTP.
2. Incubate at 94°C for 3 min, and quick-chill on ice. Spin down the reaction mixture.

3. Add 1 μL of TdT (15 U/μL) and incubate at 37°C for 10 min. Heat inactivate the TdT at 70°C for 10 min. Spin down the reaction mixture and place on ice.

3.1.4. Second-Strand cDNA Synthesis

1. Add 1 μL (500 ng) of Oligo-dG anchor primer to the TdT reaction mixture.
2. Incubate at 70°C for 10 min, and quick-chill on ice. Spin down the reaction mixture, and add the following: 100 μL sterile H_2O; 15 μL 10X Pol I buffer; 5 μL 10 m*M* dNTP mix; 5 μL *E. coli* DNA polymerase I (10 U/μL);
3. Incubate at 16°C for 2 h, then place on ice. Spin down the reaction mixture.

3.1.5. Fragmentation of Second-Strand cDNA

1. Sonicate the cDNA for 30 s in ice-water, chill on ice for 30 s, and spin down the reaction mixture. Repeat sonication for 5 times.
2. Incubate at 16°C for 5 min and add 2 μL of T4 DNA polymerase (5 U/μL). Incubate at 16°C for 5 min, then place on ice.
3. Add 150 μL of Phenol/ CIAA, vortex thoroughly, and centrifuge at room temperature for 5 min at 10,000*g*. Carefully remove 140 μL of the upper aqueous layer, and transfer it to a new 1.5-mL microcentrifuge tube.
4. Add 70 μL of 7.5 *M* NH_4OAc, followed by 500 μL of absolute EtOH (–20°C). Incubate at –20°C for 30 min. Centrifuge at room temperature for 10 min at 10,000*g*.
5. Remove the supernatant, and overlay the pellet with 0.5 mL of 70% EtOH (–20°C). Centrifuge at room temperature for 2 min at 10,000*g*, and remove the supernatant. Dry the cDNA at 37°C for 10 min to evaporate residual ethanol.

3.1.6. Adaptor Ligation

1. Add the following to cDNA pellet on ice: 5 μL sterile H_2O; 4 μL 5X T4 DNA ligase buffer; 10 μL Uni-Amp Xba I adaptor (2 μ*M*);1 μL T4 DNA ligase (1 U/μL).
2. Incubate at 16°C overnight.
3. Incubate at 70°C for 10 min, and quick-chill on ice. Spin down the reaction mixture.
4. Electrophorese on a 2% low melting agarose gel.
5. Recover the cDNA fragments ranging in size from 300–600 bp by PCR Prep Kit (Promega).
6. Elute the fragments with 50 μL of TE.

3.1.7. PCR Amplification of cDNA fragments

1. Add the following to a 0.5-mL microcentrifuge tube on ice: 5 μL cDNA fragments; 5 μL 10X PCR buffer; 3 μL 25 m*M* $MgCl_2$; 1 μL 10 m*M* dNTP mix; 1 μL UAP1 primer (10 μ*M*); 1 μL UAP2 primer (10 μ*M*); 33.5 μL Sterile H_2O.
2. Incubate at 94°C for 3 min and add 1 μL of Taq DNA polymerase (5 U/μL).
3. Perform 25 to 30 cycles of PCR amplification: Denature, 94°C for 30 s, Anneal, 60°C for 1 min; Extension, 72°C for 2 min. Incubate at 72°C for 5 min following the last cycle of PCR, then maintain at 4°C.

3.1.8. Digestion of the Amplified Fragments with Restriction Enzymes

1. Electrophorese amplified fragments on a 2% low melting agarose gel.
2. Recover the cDNA fragments ranging in size from 300–600 bp by PCR Prep Kit (Promega).
3. Elute the fragments with 50 μL of sterile H_2O.
4. Add the following: 50 μL cDNA fragments; 7 μL 10X T buffer; 7 μL 200 m*M* Spermidine; 3 μL *Sal*I; 3 μL *Xba*I.
5. Incubate at 37°C for 1 h.
6. Electrophorese on a 2% low melting agarose gel.
7. Recover the cDNA fragments ranging in size from 300–600 bp by PCR Prep Kit.
8. Elute the fragments with 50 μL of TE.

3.2. Construction Signal Sequence Trap Library

3.2.1. Preparation of the pDREF-CD4ST Vector

1. Add the following: 10 μg pDREF-CD4ST; 7.5 μL10X T buffer; 7.5 μL 200 m*M* Spermidine; Sterile H_2O to 75 μL.
2. Add 2.5 μL of *Sal*I and incubate at 37°C for 1 h. Add 2.5 μL of *Xba*I and incubate at 37°C for 1 h.
3. Purify the vector by Chromaspin TE-1000 (Clontech) spun column.
4. Add 10 μL of 10X T buffer and 10 μL of 200 m*M* Spermidine to the elute. Add 2.5 μL of *Xba*I and incubate at 37°C for 1 h. Add 2.5 μL of *Sal*I and incubate at 37°C for 1 h.
5. Purify the vector by Chromaspin TE-1000 (Clontech) spun coloumn. Analyze 5 μL of the elute on a 0.8% agarose gel.

3.2.2. Ligation of the cDNA Fragments to the pDREF-CD4ST Vector

1. Add the following to 0.5-mL microcentrifuge tubes at room temperature:

cDNA	Vector 5X ligase buffer	H_2O	T4 DNA ligase
8 μL	150 ng 4 μL	to 19 μL	1 μL
2 μL	150 ng 4 μL	to 19 μL	1 μL
0.5 μL	150 ng 4 μL	to 19 μL	1 μL
0 μL	150 ng 4 μL	to 19 μL	1 μL

2. Incubate for 3 h at room temperature.
3. Add 5 μL of yeast tRNA (1 μg/μL) and 12.5 μL of 7.5 *M* NH_4OAc.
4. Add 37.5 μL of Phenol/CIAA, vortex thoroughly, and centrifuge at room temperature for 5 min at 10,000*g*. Carefully remove 35 μL of the upper aqueous layer, and transfer it to a 0.5-mL microcentrifuge tube.
5. Add 70 μL of absolute EtOH (–20°C). Incubate at –20°C for 30 min. Centrifuge at room temperature for 10 min at 10,000*g*.

6. Remove the supernatant, and overlay the pellet with 0.5 mL of 70% EtOH (–20°C). Centrifuge at room temperature for 2 min at 10,000*g*, and remove the supernatant. Dry the cDNA at 37°C for 10 min to evaporate residual ethanol.
7. Dissolve ligated cDNA in 5 μL of sterile H_2O.

3.2.3. Introduction of Ligated cDNA into E. coli *by Electroporation*

1. Add 1 μL of the ligated cDNA or control plasmid (10 pg of pUC119) to 20 μL of ELECTROMAX DH10B cells. Put the mixture into 0.2-cm Pulser cuvet, and place on ice.
2. Electroporate at 16.6 kV/ cm, 200 ohm and 25 μF.
3. Immediately add 1 mL of S.O.C. medium, and incubate at 37°C for 1 h with vigorous aeration.
4. Plate portions of the cells on LB plate containing 100 μg/mL ampicillin. Plate the equivalent of 10, 1, and 0.1 μL, made by serial dilution into LB medium.
5. Incubate the plates overnight at 37°C, and remaining transformed cells at 4°C overnight.
6. Count the colonies on each plate, calculate the number of colonies that would result from plating the entire 1 mL of cells.
7. Plate at a 5×10^4 colonies per 150 mm LB-Amp plate.
8. Incubate the plates overnight at 37°C, and then store at 4°C.

3.2.4. Analysis of Insert DNA Fragment by PCR Amplification

1. Add the following to 0.5-mL microcentrifuge tubes: 2.5 μL 10X PCR buffer; 2 μL 2.5 m*M* dNTP mix; 100 ng EF seq F primer; 100 ng CD4 ST seq R primer; 0.1 μL Taq DNA polymerase (5 U/μL); sterile H_2O to 25 μL.
2. Pick up single colony into the reaction mixture.
3. Incubate at 94°C for 3 min. Perform 40 cycles of PCR amplification: denature, 94°C for 30 s; annealing, 55°C for 1 min; extension, 72°C for 1 min. Incubate at 72°C for 5 min following the last cycle of PCR, then maintain at 4°C.
4. Analyze 10 μL of the amplified sample on a 2% low melting agarose gel.

3.3. Signal Sequence Trap

3.3.1. Electroporation of a Signal Sequence Trap Library into Raji Cells

1. Maintain Raji cells in RPMI-1640/10% fetal calf syndrom (FCS) in a humidified 37°C, 5% CO_2 incubator. Before the day of electroporation, dilute the culture 3X to ensure that the cells are in logarithmic growth phase.
2. Wash cells with phosphate-buffered saline (PBS) twice at room temperature and suspend the cells at 1×10^7 cells per 500 μL of PBS. Add 500 μL of cell suspension and 20 μL of plasmid to 0.4-cm Pulser cuvet, and incubate at room temperature for 10 min.

3. Electroporate at 270 V-500 μF using Gene Pulser equipped with Capacitance extender (Bio-Rad). Place the cuvet at room temperature for 10 min, transfer the cells to 30 mL RPMI/10% FCS in a 75-cm^2 flask, and culture in a humidified 37°C, 5% CO_2 incubator.
4. Two days after transfection, add hygromycin to a final concentration at 200 μg/mL to obtain stable transformants. When the cells grow nearly confluent, dilute the culture 3–5× by adding RPMI1640/10% FCS/200 μg/mL hygromycin. Continue culture under the selection with 200 μg/mL hygromycin at least for a week and until total cell number becomes over 2×10^7.

3.3.2. Isolation of CD4 Positive Cells by Magnetic Cell Sorting

1. Wash cells with PBS/10% FCS twice at room temperature and suspend the cells at 2×10^7 cells/mL of PBS/10% FCS. Add OKT4 mAb to a final concentration of 10 μg/mL and incubate at room temperature for 30 min on a rotator with end-over-end rotation, at 6 to 10 rpm.
2. Wash cells to remove unbound mAb with PBS/10% FCS twice at room temperature and suspend the cells at 2×10^7 cells/mL of PBS/10% FCS.
3. During washing cells, wash magnetic beads as follows. Resuspend the magnetic beads suspension well by shaking the vial thoroughly, immediately transfer the beads suspension (25 μL [10^7 beads] per 10^7 cells) to a 15-mL polypropylene tube. Add 10 mL PBS/10% FCS, agitate, and then pull beads to the side of tube with magnetic separation apparatus. After beads have accumulated adjacent to magnet (> 1 min.), aspirate the fluid. Remove the tube from the magnetic separation apparatus, repeat washing with 10 mL PBS/10% FCS, and resuspend the beads in a volume of PBS/10% FCS equal to original beads suspension taken from the vial.
4. Add 50 μL of washed magnetic beads suspension to 1 mL of cell suspension from step 2 and incubate at room temperature for 30 min on a rotator with end-over-end rotation, at 6 to 10 rpm.
5. Place the tube in magnetic separation apparatus and allow the cells coated with magnetic beads to accumulate to the side of tube adjacent to magnet (> 1 min.). Remove the unbound cells using pasture pipet.
6. Remove the tube from the magnetic separation apparatus, carefully resuspend the cells in 10 mL PBS/10% FCS, and separate the cells coated with magnetic beads as in step 5. Repeat washing at least 3 times.
7. Resuspend cells attached to magnetic beads in RPMI/10% FCS and transfer to a 75-cm^2 flask, and culture in a humidified 37°C, 5% CO_2 incubator.
8. Two days after magnetic cell sorting, add hygromycin to a final concentration at 200 μg/mL to obtain stable transformants. When the cells grow nearly confluent, dilute the culture 3–5× by adding RPMI1640/10% FCS/200 μg/mL hygromycin. Continue culture under the selection with 200 μg/mL hygromycin until total cell number becomes over 2×10^7.
9. Determine the percentage of CD4-positive cells by staining with FITC-Leu3a and FACS.
10. Repeat **steps 1–9** until no further enrichment of CD4-positive cells is obtained.

3.3.3. Rescue of Extrachromosomal DNA from Raji Cells

1. Wash the CD4-positive cells with PBS twice at room temperature and suspend the cells at 5×10^6 cells per mL of PBS.
2. Transfer 1 mL of the cell suspension to a 1.5-mL microcentrifuge tube and centrifuge at 10,000*g* at room temperature for 10 s.
3. Remove the supernatant by vacuum aspiration, add 150 μL of solution I to the tube, and resuspend the pellet by gentle vortex. Incubate at room temperature for 5 min.
4. Add 150 μL of solution II and gently mix by inverting the tube several times. Incubate at room temperature for 5 min. Note that the suspension becomes almost translucent.
5. Add 150 μL of solution III and gently mix by inverting the tube several times. Incubate on ice for 10 min. Note that a white precipitate appears.
6. Centrifuge at 10,000*g* at 4°C for 10 min. During the centrifugation, prepare a new tube containing 0.5 mL of phenol/CIAA.
7. Transfer the supernatant to the tube containing phenol/CIAA, vortex thoroughly, and centrifuge at room temperature for 5 min at 10,000*g*. Carefully remove 400 μL of the upper aqueous layer, and transfer it to a 1.5-mL microcentrifuge tube.
8. Add 800 μL of absolute EtOH (−20°C). Incubate at −20°C for 30 min. Centrifuge at room temperature for 10 min at 10,000*g*.
9. Remove the supernatant by decantation, and overlay the pellet with 1 mL of 70% EtOH (−20°C). Centrifuge at room temperature for 2 min at 10,000*g*, Carefully decant the supernatant and respin the pellet in order to remove the last trace of liquid by pipet. Dry the cDNA at 37°C for 10 min to evaporate residual ethanol.
10. Dissolve the DNA in 5 μL of TE and re-introduce into *E. coli* by electroporation as in **Subheading 3.2.3.**

3.3.4. Identification of cDNA Clone Encoding Putative Signal Sequence

1. Pick a single, isolated colony using a sterile toothpick and inoculate them to a 1.5-mL microcentrifuge tube containing 1 mL of LB medium containing 100 μg/mL ampicillin. Place the tube in 37°C shacking incubator and incubate overnight at 250–300 rpm.
2. Centrifuge at 12,000 rpm at room temperature for 1 min, and isolate plasmid as in **steps 3–9** in **Subheading 3.3.3.**, but without phenol/CIAA extraction.
3. Dissolve the plasmid in 25 μL of TE.
4. Reintroduce 20 μL of plasmid into Raji cells by electroporation as in **steps 1–3** in **Subheading 3.3.1.** Use 2.5×10^6 cells per 250 μL of PBS, and electroporate at 270 V-250 μF. Transfer the cells to 5 mL RPMI/10% FCS in a well of a 6-well plate, and culture in a humidified 37°C, 5% CO_2 incubator.
5. Two days after transfection, examine the surface expression of the reporter CD4 antigen by staining with FITC-Leu3a and FACS and identify clones containing with putative signal sequences.

3.4. Analysis of Clones Containing Putative Signal Sequences

1. Determine the length of insert fragments as in **Subheading 3.2.4.** and sequence the fragments longer than 200 bp using with EF seq F primer.
2. Analyze nucletotide sequences and deduced amino acid sequences by computer programs for hydrophathy ***(7)***, prediction of signal sequence ***(8)***, and homology search.
3. If you find an interesting sequence, full-length cDNA can be obtained by using the fragment as a probe. Confirm that recombinant protein is secreted into culture medium from mammalian cells transfected with the full-length cDNA, and then analyze biological and biochemical property of the gene product.

4. Notes

1. To reduce false-positive clones, mRNA should be prepared free from rRNA and genomic DNA as possible. We purify and concentrate mRNA by QuickPrep Micro mRNA purification Kit (Pharmacia) using glycogen as a carrier. Then, contaminated gemonic DNA is digested with RNase-free DNase I (Gibco-BRL).
2. Excess 5' portion-enriched cDNA reduces number of colony and increases the possibility of multiple cDNA inserts into the vector. Thus, optimal concentration of the cDNA inserts must be determined.
3. In our experience, about 10^6 stable transformants were routinely obtained from 10^7 Raji cells. Therefore, in order to obtain a population that represents the full cDNA library, perform one electroporation with 10^7 Raji cells/2 × 10^5 independent *E. coli* colony.
4. Raji cells are weak to exposure to the cold environment. Therefore, we perform all the manipulation at room temperature.
5. MACS and FACS are used instead of magnetic sorting with Dynabeads.
6. After three times of expansion and sorting, CD4 antigen-positive cells were enriched from 0.1% to 44.8% (about 450-fold), in our hands.
7. Out of just 100 randomly selected clones recovered from the transformants after the third sorting, 42 clones were found to direct surface expression of CD4 antigen. To avoid analysis of cDNA fragments encoding hydrophobic sequences in unnatural open reading frames, we only sequenced 36 clones with inserts longer than 200-bp. We found that 22 clones possessed anchor oligo(dC) introduced at 5' ends of mRNA as the tag (61%). Among these 22 clones, at least 10 clones presented 5' sequences with hydrophobic profiles characteristic of signal sequence (45%). In the present experiment, 10 out of 36 trapped clones (28%) contained potential signal sequences, and the rate of trapping signal sequences in the starting *E. coli* clones was thus 10%. The analysis of false-positive clones indicated potential contamination of gemonic DNA fragments and internal fragments of cDNA. These problems would be reduced by treatment of mRNA with DNase and by selective labeling of 5' ends of mRNA by replacing the cap structure with an oligonucleotide ***(10)***.

8. We have obtained signal sequences of transmembrane proteins (CD45, Leu-8, CD6, MGC-24, β2-microglobulin, TCR-ε, granule proteoglycan), secreted proteins (TARC, SCM-1/lymphotactin) ***(2,11)***, and proteins accumulated in intracellular organelles (NAcGlu-1-P-transferase).
9. Recently, signal sequence selection methods in yeast have also been described ***(12,13)***.

References

1. Tashiro, K., Tada, H., Heilker, R., Shirozu, M., Nakano, T., and Honjo, T. (1993) Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. *Science* **261,** 600–603.
2. Imai, T., Yoshida, T., Baba, M., Nishimura, M., Kakizaki, M., and Yoshie, O. (1996) Molecular cloning of a novel T cell-directed CC chemokine expressed in thymus by signal sequence trap using Epstein-Barr virus vector. *J. Biol. Chem.* **271,** 21,514–21,521.
3. Tashiro, K., Nakano, T., and Honjo, T. (1997) Signal sequence trap. Expression cloning method for secreted proteins and type 1 membrane proteins. *Methods Mol. Biol.* **69,** 203–219.
4. Frohman, M. A., Dush, M. K., and Martin, G. R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85,** 8998–9002.
5. Yates, J. L., Warren, N., and Sugden, B. (1985) Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. *Nature* **313,** 812–815.
6. Margolskee, R. F., Kavathas, P., and Berg, P. (1988) Epstein-Barr virus shuttle vector for stable episomal replication of cDNA expression libraries in human cells. *Mol. Cell. Biol.* **8,** 2837–2847.
7. Kyte, J. and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157,** 105–132.
8. von Heijne, G. (1986) A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* **14,** 4683–4690.
9. Mizushima, S. and Nagata, S. (1990) pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* **18,** 5322.
10. Maruyama, K. and Sugano, S. (1994) Oligo-capping: a simple method to replace the cap structure of eukaryotic mRNAs with oligoribonucleotides. *Gene* **138,** 171–174.
11. Yoshida, T., Imai, T., Kakizaki, M., Nishimura, M., and Yoshie, O. (1995) Molecular cloning of a novel C or gamma type chemokine, SCM-1. *FEBS Lett.* **360,** 155–159.
12. Klein, R. D., Gu, Q., Goddard, A., and Rosenthal, A. (1996) Selection for genes encoding secreted proteins and receptors. *Proc. Natl. Acad. Sci. USA* **93,** 7108–7113.
13. Jacobs, K. A., Collins-Racie, L. A., Colbert, M., Duckett, M., Golden-Fleet, M., Kelleher, K., et al. (1997) A genetic selection for isolating cDNAs encoding secreted proteins. *Gene* **198,** 289–296.

3

Chemokine Expression in Insect Cells

Toshio Imai

1. Introduction

Recent progress in the collection of cDNA sequences by random sequencing reveals numerous genes of biological interest. Although a putative function may be predicted from deduced amino acid sequence, a rigorous proof of identity of a gene will require a demonstration of the appropriate biological activities of gene products. Thus, efficient expression of recombinant proteins of cloned genes is essential to obtain proteins sufficient for detailed biological and biochemical analysis. In this chapter, I will describe a rapid and efficient method to generate recombinant chemokines in insect cells ***(1,2)***.

Recombinant baculoviruses have been used as vectors to express heterologous proteins in cultured insect cells ***(3)***. A number of unique features distinguish the baculovirus expression system from other expression systems;

1. High levels of heterologous gene expression are often achieved by using the strong polyhedrin promoter of the *Astographa californica* nuclear polyhedrosis virus (AcNPV);
2. Most mammalian signal sequences are correctly processed and the recombinant proteins are efficiently secreted into culture medium;
3. The secreted recombinant proteins are easily detected and purified from serum-free medium of infected cells late in infection when host protein synthesis is diminished.

Because AcNPV is a large (130 kb) circular double-stranded DNA virus, direct cloning of heterologous genes into the genome is hampered. Traditionally, recombinant baculoviruses are constructed by homologous recombination between a plasmid transfer vector containing a gene to be expressed flanked by a portion of baculovirus DNA and wild-type viral DNA in insect

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cells ***(3)***. The resulting progeny viruses are mixture of wild-type and recombinant viruses, but typically 0.1 to 1% of the progeny viruses are recombinant. The fraction of recombinant virus can be improved to over 80% by using linearized viral DNA that is missing an essential portion of the baculovirus genome for replication ***(4,5)***. In these cases, sequential plaque assay or limiting dilution is required to isolate the desired recombinant virus from the nonrecombinant wild-type virus. The desired recombinant virus is identified in a variety of ways, including visual screening of occlusion-negative plaque phenotype, filter hybridization with the gene to be expressed, and immunological screening with specific antibody. Although the baculovirus expression system is a powerful system for heterogeneous gene expression, isolation and identification of a recombinant virus take more than a month to complete.

Recently, a rapid and efficient method to generate recombinant baculoviruses was developed utilizing site-specific transposition of a mini-Tn7 element from a donor plasmid (pFastBac1) into a mini-*att*Tn7 attachment site on a baculovirus shuttle vector (bacmid) propagated in *Escherichia coli* ***(6,7)***. Genes to be expressed are inserted into the donor plasmid (pFastBac1), downstream of the polyhedrin promotor. The resultant transfer vector contains a mini-Tn7 expression cassette consisting of a gentamicin-resistance gene, the polyhedrin promotor, the desired gene in a multiple cloning site, and an SV40 poly(A) signal inserted between the left and right arms of Tn7. The transfer vector is transformed into *E. coli* DH10Bac containing a bacmid (bMON14272) consisting of a kanamycin-resistance gene, baculovirus genome, and a mini-*att*Tn7 attachment site inserted N-terminus of the *lac*Z alpha gene without disruption of the reading frame of the *lac*Z alpha peptide, and a helper plasmid (pMON7124) that provides Tn7 transposition functions *in trans*. The mini-Tn7 expression cassette on pFastBac1 transposes into the mini-*att*Tn7 attachment site on the bacmid in the *E. coli* DH10Bac. Insertions of the mini-Tn7 into the mini-*att*Tn7 target site on the bacmid disrupts expression of the the *lac*Z alpha peptide, so colonies containing the recombinant bacmid are white in a background of blue colonies that propagate the unaltered bacmid in the presence of X-gal. Recombinant bacmid DNA is easily isolated from small scale cultures by alkaline lysis method, transfected into Sf9 cells using the CellFectin reagent, and the recombinant viruses are obtained. Since recombinant bacmid DNA isolated from single colony is not mixed with nonrecombinant virus, recombinant virus harvested from the transfected cells is genetically identical. As a result, the progeny virus in the supernatant of the transfected cells can be used to infect fresh insect cells for subsequent protein expression, purification, and analysis. This method eliminates the need for identification and isolation of a

recombinant virus and thus greatly reduces time to obtain desired recombinant virus from at least 4 wk to within 1 wk. This method also permits the rapid and simultaneous isolation of multiple recombinant virus with greatly reduced labor.

2. Materials

2.1. Reagents

1. Bac-to-Bac Baculovirus Expression System (Gibco-BRL): plasmid pFastBac1, MAX Efficiency DH10Bac competent cells, and CellFECTIN reagent.
2. For insect cell culture: *Spodoptera frugiperda* Sf9 cells (ATCC-CRL-1711), High Five cells (BTI-TN-5B1-4; Invitrogen); TNM-FH medium (supplemented Grace's insect cell culture medium, Gibco-BRL): Grace's insect cell culture medium containing Lactoalbumin hydrolysate and Yeastlate; EX-CELL 400 serum free medium (JRH Bioscience); Fetal calf serum (FCS, HyClone Logan, UT); Dimethylsulfoxide (DMSO) (cell culture grade).
3. LB Agar plates containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 100 μg/mL X-gal, and 40 μg/mL IPTG.
4. For isolation of bacmid DNA: Solution I: 15 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, 100 μg/mL RNase A, 1 mg/mL lysozyme. Filter-sterilize and store at 4°C. Solution II: 0.2 *N* NaOH; 1% SDS. Filter-sterilize and store at room temperature. Solution III: 3 *M* KOAc. Autoclave and store at 4°C.
5. Other reagents: SOC medium (Gibco-BRL); TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA; Phenol/CIAA saturated with TE; 7.5 *M* NH_4OAc; EtOH; 70% EtOH; sterile water; SDS-PAGE gel; and silver-staining kit.

2.2. Equipment

1. Cell scrapers (Nunc).
2. Water bath capable of maintaining 42°C.
3. Incubator capable of maintaining 27°C (CO_2 not required) for growing insect host cells.
4. Inverted microscope with 10×, 20×, and 40× objectives to monitor monolayer cultures and to perform cell counts.
5. Laminar flow-hood for tissue culture.
6. Liquid nitrogen storage facility.
7. Tissue culture flask (25 cm^2, 75 cm^2, and 150 cm^2) for adherent cells to maintain High Five cells and to infect baculovirus to Sf9 cells and High Five cells.
8. Tissue culture flask (25 cm^2, 75 cm^2, and 150 cm^2) for suspension cells to maintain Sf9 cells.
9. Polystyrene test tubes used for bacmid transfection. CellFECTIN-DNA complex stick to the walls of polypropylene tubes, so polystyrene should be used.

3. Methods

3.1. Cell Culture of Insect Cells

3.1.1. Establishing Sf9 Cells From Stock

1. Add 5 mL of cold (4°C) TNM-FH;10% FCS to a 25-cm^2 flask.
2. Remove vial from liquid nitrogen and partly thaw rapidly in a 37°C waterbath with gentle agitation.
3. Place the vial on ice until the cell suspension is completely thawed.
4. Rinse the vial with 70% EtOH to decontaminate the outside.
5. Transfer the cell suspension to the prepared flask.
6. Incubate for 1–3 h at 27°C to allow the cells to attach.
7. Remove the medium and unattached cells and replace with 5 mL of fresh TNM-FH/10% FCS.
8. Incubate at 27°C until the cells have formed 80–100% confluent monolayer.

3.1.2. Maintenance of Sf9 Cells

1. Remove medium and floating cells from a confluent monolayer.
2. Add 5 mL of TNM-FH,10% FCS to the 25-cm^2 flask.
3. Gentry dislodge the cells by a cell scraper, and disperse by gently pipeting up and down several times with a 5-mL plastic disposable pipet.
4. Add 4 mL of TNM-FH/10% FCS to a fresh 25-cm^2 flask for suspension cells and add 1 mL of the cell suspension (approx 10^6 cells/25-cm^2 flask).
5. Incubate for 4–5 d in a 27°C incubator.
6. If the monolayer does not reach 80–100% confluency, remove the spent medium from one side of the monolayer and gently refeed with 5 mL of fresh TNM-FH,10% FCS. Incubate for a few more days in a 27°C incubator.
7. Subculture the flask as described above in **steps 1–4** when the monolayer reaches 80–100% confluency.

3.1.3. Cryopreservation of Sf9 Cells

1. Monitor and choose healthy mid to late log-phase culture (80–90% confluency, > 90% viability).
2. Gently dislodge the cells by a cell scraper, and disperse by gently pipeting up and down several times with a 5-mL plastic disposable pipet.
3. Harvest the cells to a 15-mL tube and centrifuge at 300*g* at room temperature for 5 min.
4. Resuspend the cells to a density of 3×10^6 cells/mL in ice-cold freshly prepared Sf9 freezing medium (80% TNM-FH;10% FCS;10% DMSO, filter-sterilized).
5. Dispense 1 mL of cell suspension into cryogenic vials and cap tightly.
6. Place vials at –20°C for 1 h, at –80°C for 24 h, and finally transfer to liquid nitrogen storage.

3.1.4. Establishing of High Five Cells From Stock

1. Add 5 mL of cold (4°C) Ex-Cell 400 serum-free medium to a 25-cm^2 flask.
2. Remove vial from liquid nitrogen and partly thaw rapidly in a 37°C waterbath with gentle agitation.
3. Place the vial on ice until the cell suspension is completely thawed.
4. Rinse the vial with 70% EtOH to decontaminate the outside.
5. Transfer the cell suspension to the prepared flask.
6. Incubate for 20 min to 1 h at 27°C to allow the cells to attach.
7. Remove the medium and unattached cells and replace with 5 mL of fresh Ex-Cell 400.
8. Incubate at 27°C until the cells have formed 80–100% confluent monolayer.

3.1.5. Maintenance of High Five Cells

1. Remove medium and floating cells from a confluent monolayer.
2. Add 5 mL of Ex-Cell 400 serum-free medium to the 25-cm^2 flask.
3. Resuspend cells by pipeting the medium across monolayer with a 5-mL plastic disposable pipet.
4. Add 4 mL of Ex-Cell 400 to a fresh 25-cm^2 flask for adherent cells and add 1 mL of the cell suspension.
5. Incubate for 3–4 d in a 27°C incubator.
6. If the monolayer does not reach 80–100% confluency, remove the spent medium from one side of the monolayer and gently refeed with 5 mL of fresh Ex-Cell 400. Incubate for a few more days in a 27°C incubator.
7. Subculture the flask as described in **steps 1–4** when the monolayer reaches 80 to 100% confluency.

3.1.6. Cryopreservation of High Five Cells

1. Monitor and choose healthy mid to late log-phase culture (80–90% confluency, > 90% viability).
2. Resuspend cells by pipeting the medium across monolayer with a 5-mL plastic disposable pipet.
3. Harvest the cells to a 15-mL tube and centrifuge at 300*g* at room temperature for 5 min. Recover conditioned medium to prepare High Five freezing medium.
4. Resuspend the cells to a density of 3×10^6 cells/ mL in ice-cold freshly prepared High Five freezing medium (45% conditioned medium; 45% fresh Ex-Cell 400 medium; 10% DMSO, filter-sterilized).
5. Dispense 1 mL of cell suspension into cryogenic vials and cap tightly.
6. Place vials at –20°C for 1 h, at –80°C for 24 h, and finally transfer to liquid nitrogen storage.

3.2. Construction of Recombinant Bacmid DNA

3.2.1. Cloning cDNA into the Donor Vector pFastBac1

1. Clone a DNA fragment encoding a foreign protein into appropriate sites of pFastBac1 vector in correct orientation with respect to the polyhedrin promotor. The fragment must contain its own initiator codon followed by an open reading frame and terminator codon. Prepare recombinant donor plasmid DNA from *E. coli* using the standard procedure (e. g., alkaline lysis method).

3.2.2. Transposition in E. coli

1. Prepare LB Agar plates containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 100 μg/mL X-gal, and 40 μg/mL IPTG.
2. Thaw the DH10Bac competent cells on ice. Dispense 20–100 μL of the cells into 15-mL polypropylene tube.
3. Dilute the recombinant donor plasmid DNA from **Subheading 3.2.1.** with TE at 1 ng/μL. Add 1 μL of diluted plasmid DNA to the tube containing competent cells and gently mix by tapping.
4. Incubate the mixture on ice for 30 min.
5. Heat-shock the mixture by placing the tube in 42°C water bath for 45 s.
6. Chill the mixture on ice for 2 min.
7. Add 900 μL of SOC medium to the mixture.
8. Place the tube in 37°C shacking incubator with gentle agitation and incubate for 4 h.
9. Spread 10 μL over a plate and 100 μL over another plate.
10. Incubate at 37°C for at least 24 h or until blue colonies are clearly identified.

3.2.3. Isolation of Recombinant Bacmid DNA

1. Select the largest and most isolated white colonies from the plates to avoid the selection of false positives and possible cross-contamination.
2. Streak several candidate colonies to fresh plates prepared as in **Subheading 3.2.2., step 1** to evaluate the phenotype and cross contamination. Incubate overnight at 37°C
3. Pick a single, isolated white colony from each plate using a sterile toothpick and inoculate it to a 15-mL polypropylene tube containing 3 mL of LB medium supplemented with kanamycin (50 μg/mL), gentamicin (7 μg/mL), and tetracycline (10 μg/mL). Place the tube in 37°C shacking incubator and incubate overnight at 250 to 300 rpm.
4. Transfer 1.5 mL of culture to a 1.5-mL microcentrifuge tube and centrifuge at 1000*g* at room temperature for 1 min.
5. Remove the supernatant by vacuum aspiration, add 0.3 mL of solution I to the tube, and resuspend the pellet by gentle vortex. Incubate at room temperature for 5 min.
6. Add 0.3 mL of solution II and gently mix by inverting the tube several times.

Incubate at room temperature for 5 min. Note that the suspension becomes almost translucent.
7. Dropwise add 0.3 mL of solution III and gently mix by inverting the tube several times. Incubate on ice for 10 min. Note that a white precipitate will appear.
8. Centrifuge at 10,000*g* at 4°C for 10 min. During the centrifugation, prepare a new tube containing 0.8 mL of isopropanol.
9. Carefully transfer the supernatant to the tube containing isopropanol and gently mix by inverting the tube several times. Incubate on ice for 10 min.
10. Centrifuge at 10,000*g* at room temperature for 15 min.
11. Carefully pour off the supernatant and add 1 mL of 70% EtOH. Wash the pellet by inverting the tube several times. Centrifuge at 10,000*g* at room temperature for 5 min.
12. Carefully pour off as much of the supernatant as possible and place the tube on kimwipe at inverted position for 5 min at room temperature to dry the pellet.
13. Add 40 μL of TE and dissolve the bacmid DNA by incubating at room temperature for 30 min. Do not use pipeting or vortex for resuspension of the bacmid DNA to avoid mechanical shearing.
14. Store at 4°C until use.

3.3. Preparation of Recombinant Baculovirus

3.3.1. Transfection of Sf9 Cells with Recombinant Bacmid DNA

1. Add 9×10^5 Sf9 cells in 2 mL of TNM-FH;10% FCS per 35-mm well of a 6-well plate, and incubate at 27°C for 1 h to allow the cells to attach.
2. Prepare the following solutions in 12 × 75-mm sterile polystyrene tubes: Tube 1: Add 5 μL of Bacmid DNA from **Subheading 3.2.** to 100 μL of serum-free TNM-FH; Tube 2: Add 5 μL of CellFECTIN reagent to 100 μL of serum-free TNM-FH.
3. Transfer the solution of Tube 2 into Tube 1, mix gently, and incubate at room temperature for 15 to 45 min.
4. Remove medium from the well from **step 1** by vacuum aspiration, and rinse with 2 mL of serum-free TNM-FH. Add 2 mL of serum-free TNM-FH and incubate at room temperature for 10 min.
5. Add 0.8 mL of serum-free TNM-FH to the tube from **step 3**, and mix gently. Remove wash medium from the well by vacuum aspiration and add the transfection mixture.
6. Incubate for 5 h in a 27°C incubator.
7. Remove the transfection mixture by vacuum aspiration and rinse with 2 mL of TNM-FH,10% FCS. Add 2 mL of TNM-FH,10% FCS.
8. Incubate for 3 d in a 27°C incubator.
9. Harvest the supernatant to a 15-mL tube and centrifuge at 300*g* at room temperature for 5 min.
10. Collect the supernatant to a fresh tube and label as (Passage 1) (P1) virus. Store the virus in a dark place at 4°C. For long term storage, store an aliquot of the virus at –70°C.

3.3.2. Analysis of Expression by Recombinant Baculoviruses

1. Add 2 × 10^6 High Five cells to a 25-cm^2 flask and incubate at 27°C for 1 h to allow the cells to attach.
2. Remove medium by vacuum aspiration, and add 0.4 mL of Ex-Cell 400 medium. Add 0.1 mL of P1 virus stock (approx 2–4 × 10^7 pfu/mL) or appropriate control wild-type virus (approx multiplicity of infection [MOI] of 1 to 10).
3. Incubate at room temperature for 1 h on a rocking platform.
4. Remove the inoculate and rinse with 2 mL of Ex-Cell 400 medium.
5. Add 5 mL of Ex-Cell 400 medium and incubate at 27°C for 2–3 d.
6. Collect culture supernatants to a 15-mL tube. Add 5 mL of fresh Ex-Cell 400 medium to the flask, harvest the cells by pipeting, and transfer to another 15-mL tube.
7. Centrifuge both tubes at 300*g* at room temperature for 5 min.
8. For the supernatants: collect the supernatant to a 15-mL tube and transfer 1 mL to a 1.5-mL microcentrifuge tube. Add 100 µL of 90% TCA to 1 mL of supernatant and incubate on ice for 30 min. Centrifuge at 10,000*g* at 4°C for 10 min, rinse the pellet with ice-cold Acetone three times, and dry up the pellet at room temperature. Add 45 µL of SDS-PAGE buffer and 5 µL 1*M* Tris-HCl, pH 8.0, and boil for 3 min. Electrophorese 5 µL (equivalent for 100 µL of supernatants) on 10–25% SDS-PAGE gradient gels.

   For the cells: remove the supernatant and add 500 µL of PBS to the pellet. Resuspend the cells and transfer to a 1.5-mL microcentrifuge tube, centrifuge 20 µL of them, and add 40 µL of SDS-PAGE buffer. Boil and sonicate the sample. Electrophorese 20 µL (equivalent for 1/50 total cells)
9. Run samples on 10–25% gradient SDS-PAGE gels and detect by silver staining.
10. Identify a band that is present in recombinant, but absent in wild-type. If expressed protein is not secreted into the medium, a novel band appears in a lane of cell pellet but not in supernatants.

3.3.3. Preparation of Working Virus Stocks (Passage 2 [P2] virus)

1. Add 1.2 × 10^7 Sf9 cells in 10 mL of TMN-FH,10% FCS to a 150-cm flask and incubate at 27°C for 1 h.
2. Remove supernatants by vacuum aspiration. Add 5 mL TMN-FH,10% FCS and 10 µL of P1 virus solution (approx 2–4 × 10^7 pfu/mL) per flask.
3. Incubate at room temperature for 1 h on a rocking platform.
4. Remove the inoculate and add 30 mL of TMN-FH,10% FCS.
5. Incubate for 4 d in a 27°C incubator.
6. Collect culture supernatants to a 50-mL tube.
7. Centrifuge both tubes at 300*g* at room temperature for 5 min.
8. Collect the supernatant to a fresh tube and label as Passage 2 (P2) virus. Store the virus in a dark place at 4°C.

3.4. Large-Scale Recombinant Protein Production

1. Add 1.2 × 10^7 High Five cells in 10 mL of Ex-Cell 400 serum-free medium to a 150-cm flask and incubate at 27°C for 1h.

2. Remove supernatants by vacuum aspiration. Add 4 mL of Ex-Cell 400 and 1 mL of P2 virus solution (approx 1–2 × 10^8 pfu/mL) to the flask.
3. Incubate at room temperature for 1 h on a rocking platform.
4. Remove the inoculate and rinse with 5 mL of Ex-Cell 400 medium.
5. Add 30 mL of Ex-Cell 400 medium and incubate at 27°C for 48 h.
6. Collect culture supernatants, filtrate through a 0.22-μm filter, and store at 4°C for purification.

4. Notes

1. Insect cells will grow reasonably well at any temperature from room temperature to 30°C and they do not require CO_2. An optimal growth temperature is 27°C. In order to maintain consistency of cell growth and virus infection, cells should be used within 20–30 passages. After that, a culture should be replaced by retrieving a fresh batch of cells from liquid nitrogen.
2. To prevent accidental virus infection of cell cultures, all reagents should be separately prepared for noninfected cells and infected cells.
3. Although baculovirus gives a very restricted host range, wild-type and recombinant baculovirus should be treated as potential biohazards. All materials must be either detachment or disinfected before disposal.
4. Sf9 cells are very fragile for vigorous scraping or pipeting. High-Five cells are relatively resistant for pipeting and easily detached with pipeting when cells are nearly confluent. But in subconfluent, both cells are strongly attached to the flask and detachment of these cells may reduce the viability.
5. Usually, we do not determine the titer of recombinant virus, since this protocol works well for over 10 chemokines. Successful infection is easily confirmed by the morphology of the High Five cells. Uninfected cells are firmly attached and infected cells become round up. If you do not detect the expression of recombinant protein, you should determine the titer of the recombinant virus, and should optimize MOI and timing of harvest.
6. We prefer large-scale production of recombinant protein by infection in monolayer culture, since it is easier to achieve synchronous infections in monolayer culture than in suspension culture.
7. We usually transfect only two recombinant bacmid DNA obtained from independent clones. We always obtain desired recombinant virus from both.
8. If you detect accumulation of the expressed protein in the cells, but not the supernatant, the signal sequence of the protein does not work in insect cells. In my experience, ELC does not secrete from insect cells ***(8)***. In these cases, consider using other expression systems.
9. If you desire simultaneous isolation of multiple recombinant viruses, you may use 24-well culture instead of 6-well.

References

1. Imai, T., Yoshida, T., Baba, M., Nishimura, M., Kakizaki, M., and Yoshie, O. (1996) Molecular cloning of a novel T cell-directed CC chemokine expressed in

thymus by signal sequence trap using Epstein-Barr virus vector. *J. Biol. Chem.* **271,** 21,514–21,521.
2. Imai, T., Hieshima, K., Haskell, C., Baba, M., Nagira, M., Nishimura, M., Kakizaki, M., Takagi, S., Nomiyama, H., Schall, T. J., and Yoshie, O. (1997) Identification and molecular characterization of fractalkine receptor CX_3CR1, which mediates both leukocyte migration and adhesion. *Cell* **91,** 521–530.
3. O'Reilly, D. R., Miller, L. K., and Luckow, V. A., (1992) *Baculovirus Expression System: A Laboratory Manual*. W. H. Freeman & Co., NY.
4. Kitts, P. A. and Possee, R. D. (1993) A method for producing recombinant baculovirus expression vectors at high frequency. *Biotechniques* **14,** 810–817.
5. Kitts, P. A. (1995) Production of recombinant baculoviruses using linearized viral DNA. *Methods Mol. Biol.* **39,** 129–142.
6. Luckow, V. A., Lee, S. C., Barry, G. F., and Olins, P. O. (1993) Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J. Virol.* **67,** 4566–4579.
7. Luckow, V. A. (1993) Baculovirus systems for the expression of human gene products. *Curr. Opin. Biotechnol.* **4,** 564–572.
8. Yoshida, R., Imai, T., Hieshima, K., Kusuda, J., Baba, M., Kitaura, M., Nishimura, M., Kakizaki, M., Nomiyama, H., and Yoshie, O. (1997) Molecular cloning of a novel human CC chemokine EBI1-ligand chemokine that is a specific functional ligand for EBI1, CCR7. *J. Biol. Chem.* **272,** 13,803–13,809.

4

Expression of Chemokines in *Escherichia coli*

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1. Introduction

Escherichia coli is one of the most powerful and versatile hosts for high-level protein production. Its well-characterized genetics and biochemistry have led to the development of many different systems for heterologous protein expression ***(1,2)***. In this chapter, we focus on the use of the T7 expression system ***(3,4)*** as we have found this system to produce protein most consistently. Many different vectors and host strains have been developed for use with the T7 system. In the format we most commonly use, employing the vector pET24d (**Fig. 1**), transcription originates from a T7*lac* promoter ***(5)*** in response to induction of a chromosomally encoded T7 RNA polymerase gene. Inclusion of a lac operator in the T7 promoter and the presence of a vector encoded lactose repressor, lacI, helps to reduce basal transcription levels. Background expression can be further reduced by cotransformation with plasmids encoding T7 lysozyme such as plysS ***(6)***. Kanamycin selection due to intracellular expression of aminoglycoside 3'-phosphotransferase by pET24d allows more stringent selection for plasmid maintenance than if a secreted β-lactamase is used. Tight control of expression and the continued application of selective pressure are important for high-level protein production as leaky expression can select for lower expressing clones ***(7)*** and high-level protein expression produces a strong negative selection pressure, even when the expressed proteins are nontoxic ***(8)***.

For many chemokines (IL-8, MCP-3, MIP-1α and murine RANTES, **Fig. 2**), simply inserting cDNA encoding the mature chemokine into the pET vector results in high-level expression of the mature protein. However, in

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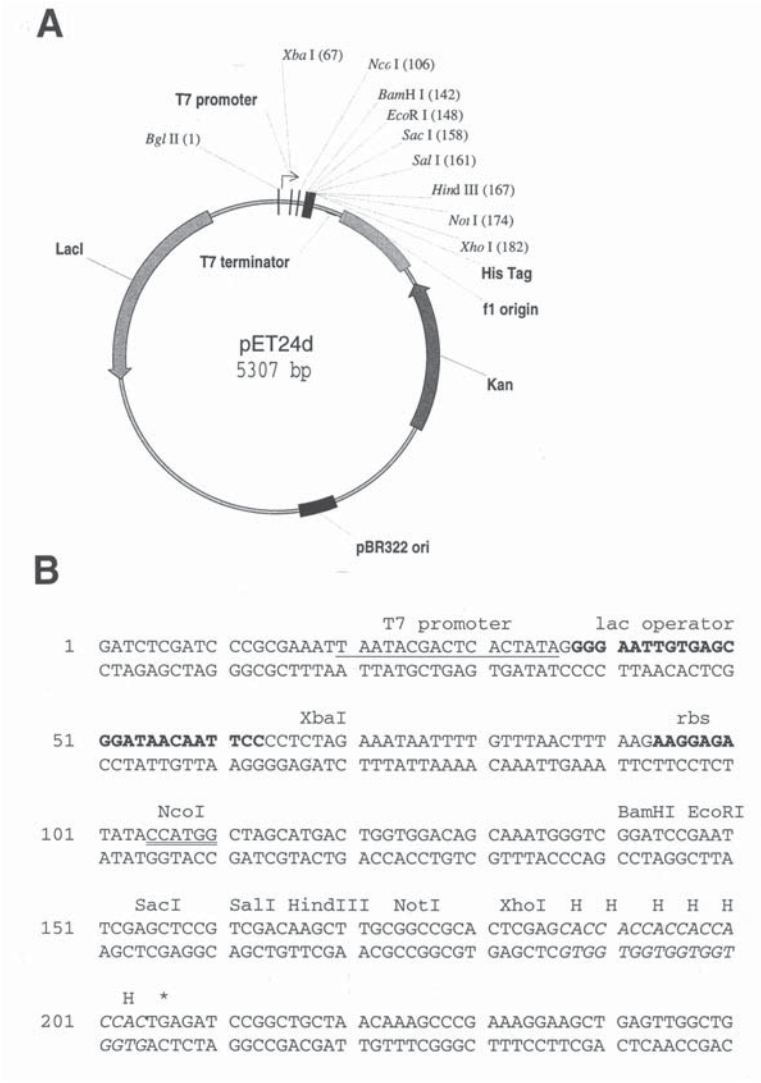

Fig 1. **(A)** T7-based E. coli expression vector pET24d. **(B)** Polylinker region of pET24d.

other cases (MCP-1, eotaxin and vMIP-IB, **Fig. 2**), no or only very little expression is seen. Failure of expression is likely to occur at the level of translation, as transcription is not usually rate-limiting in the T7 system ***(9)***. True understanding of the root causes for translational failure is limited. In some cases, secondary structure in the resulting mRNA ***(10)*** or the presence of

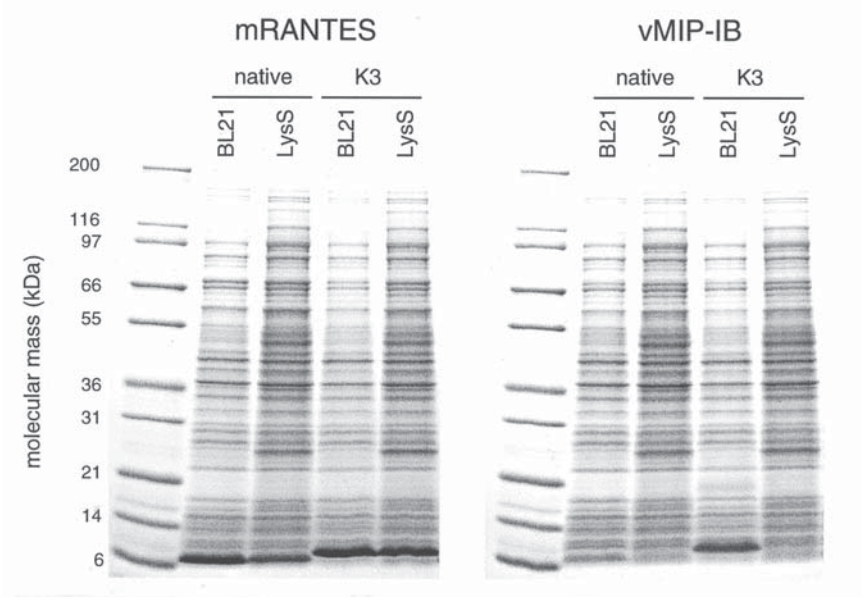

Fig 2. Expression of chemokines with and without N-terminal tags. Murine RANTES and human herpesvirus 8 vMIP-1B were expressed either as mature proteins (native) or with the addition of the residues MKKKWPR- to the N-terminus (K3) using the T7 based expression vector pET24d in either BL21(DE3) or BL21(DE3)pLysS cells. Each lane of this Coomassie stained SDS-polyacrylamide gel contains 0.04 OD_{600} units of the indicated strain following a 3-h induction.

codons rarely used in *E. coli* ***(11)***, particularly near the 5' end of the gene, have both been shown to limit translation of heterologous proteins.

On those occasions where we fail to see protein expression from the mature cDNA, two empirical steps are taken to remedy the problem. First, we add a cleaveable amino-terminal peptide tag to the protein. This tag was first developed by PeproTec for the overexpression of RANTES (S. Findley, personal communication). In its original form (MKKKWPR-), the tag was designed to include an adenine-rich enhancer motif that encodes the lysine residues ***(12)***, an ultraviolet (UV)-absorbing tryptophan residue, a proline spacer, and an arginine residue for removal of the tag with endo-argC. This worked well for RANTES. However, all other chemokines tested (MIP-4, HCC1, eotaxin, MIP-1α) were digested at internal arginine residues. We are now using two variants of this tag which replace the arginine residue by either a factor Xa cleavage site (MKKKWPIEGR) or a methionine residue (MKKKWPM) for cleavage with cyanogen bromide (**Table 1**). With the latter tag, it is necessary to mutate any internal methionines to the structurally similar leucine. In

Table 1
N-Terminal Tags Used in Expression of Chemokines

Cleavage	Sequence									
	M	K	K	K	W	P	R			
endo-argC	ATG	AAA	AAA	AAA	TGG	CCG	CGT			
	M	K	K	K	W	P	M			
cyanogen bromide[a]	ATG	AAA	AAA	AAA	TGG	CCG	ATG			
	M	K	K	K	W	P	I	E	G	R
factor Xa	ATG	AAA	AAA	AAA	TGG	CCG	ATC	GAA	GGT	CGT

[a]Requires mutation of internal methionine residues.

addition to improving expression levels of some chemokines, the N-terminal tags also allow strict control of amino-terminal residues. RANTES was found to retain its initiating methionine residue when overexpressed in the T7 system. This minor alteration had profound effects on the protein's activity ***(13)***. Likewise, we have found that about 15% of expressed MIP-1α retains its initiator methionine. We have used the endo-argC cleavable N-terminal tag as a means of producing mature RANTES, lacking the initiator methionine. As specific N-terminal residues have been shown to be important for activity in many chemokines ***(14,15)***, precise control of N-terminal residues is important.

In cases where no expression is seen with added N-terminal tags, we change codon usage in the chemokine genes. We have found that small changes in codon usage can lead to dramatic improvements in expression yields ***(16)***. However, this is a trial and error process. It is easier to change both codon usage and mRNA secondary structure at the same time by synthesizing altered versions of the gene, both with and without the amino-terminal tag, using codons found in highly expressed *E. coli* genes ***(17)***. Synthetic versions of HCC-1, MCP-4 and eotaxin were all found to express very well in the T7 system.

The pET system coupled with removable N-terminal tags has allowed us to express most chemokines in reasonable quantities. However, high-level expression is usually achieved again by a trial and error process involving changing bacterial strains (i.e., BL21[DE3], BL21[DE3]pLysS, or other DE3 lysogens [Novagen]), media (LB, terrific broth, 2X YT ***(18)***, induction times (1–4 h), growth temperature (30°C) or even expression vectors (i.e., pET23d, which has the T7 promoter rather than the T7*lac* promoter found in pET24d). This optimization process can be time-consuming, but frequently results in significant yield improvements that greatly aid downstream purification.

We have used the T7 system to express IL-8, MCP-1, MCP-3, MIP-1α, MIP-4, RANTES, HCC-1, eotaxin, and vMIP-IB, all of which were expressed as

insoluble inclusion bodies. As chemokines are usually easy to refold, and inclusion bodies simplify purification (and may protect the proteins from proteolysis), we view this as an advantage. However, other systems, notably glutathione-s-transferase (GST) fusion proteins ***(19,20)***, have been used to express soluble chemokines *E. coli*. Depending upon the intended use of the proteins, other systems may offer some advantages.

2. Materials

2.1. Cloning of the Chemokine Gene into pET24d

1. pET expression vectors (Novagen).
2. host strains: BL21(DE3) *ompT* $hsdS_B(r_B-m_B-)$ *gal dcm* (DE3); BL21(DE3)pLysS *ompT* $hsdS_B(r_B-m_B-)$ *gal dcm* (DE3)pLysS; DE3 is a λ lysogen that expresses T7 RNA polymerase from a lac promoter.

2.2. Small-Scale Expression Trials

1. Competent BL21(DE3) and BL21(DE3)pLysS cells.
2. 1*M* IPTG (isopropyl-β-thiogalactopyranoside) (Sigma).
3. 1X SDS sample buffer: 60 m*M* Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 0.025% bromophenol blue.

3. Methods

3.1. Cloning of the Chemokine Gene into pET24d

We routinely clone chemokines using polymerase chain reaction (PCR). The small size of chemokine genes, the frequent addition of N-terminal or C-terminal tags, and the need to localize precisely the initiator methionine codon in bacteria expression systems are all most easily dealt with by amplifying the genes. In this section, it is assumed that the reader has a grasp of basic cloning techniques ***(18)*** and is familiar with simple PCR amplification ***(21)***.

1. PCR primers are designed that allow the target gene to be amplified either from a cDNA clone or from single-stranded cDNA. Because pET24d requires the start codon to be compatible with an *Nco*I site, the restriction site placed at the 5' end of the gene must leave a CATG 5' overhang. *Nco*I works well here. However, it requires that the second codon begin with guanine (must encode V, A, D, E, or G). Should the mature protein begin with a different amino acid or contain an internal *Nco*I site, the *Nco*I compatible enzymes *Alf*III or *Bsp*HI may be used. Alternatively, *Bsm*BI or *Bsa*I can be used to leave *Nco*I-compatible overhangs (**Table 2**). We have found that all of these enzymes digest PCR products reasonably well when a six base tag is included at the 5' end of the primer. Other vectors in the pET series (i.e., pET24a-c) use *Nde*I overhangs at the start position. However, we have occasionally had problems digesting PCR products with this enzyme.

Table 2
Restriction Endonucleases Leaving 5' Overhangs Compatible With *Nco*I

Enzyme	Recognition site
*Nco*I	5' CCATGG 3'
*Bsp*HI	5' TCATGA 3'
*Alf*III[a]	5' ACRYGT 3'
*Bsm*BI[b]	5' CGTCTC(1/5) 3'
*Bsa*I[b]	5' GGTCTC(1/5) 3'

[a]R, A or G; Y, T or C.
[b]Can be used to leave any 4 base 5' overhang.

2. PCR reactions should be performed using a thermostable DNA polymerase with proofreading function, i.e., Vent, New England Biolabs Beverly, MA, or Pfu, Stratagene, La Jolla, CA. The protocol used will vary with the source of target DNA and enzyme used.
3. Any non-T7 expressing *E. coli* strain may be used for cloning. We routinely use DH10B [*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K1 *rps*L *nup*G] (Gibco-BRL).
4. All genes cloned by PCR must be sequenced. Even proofreading enzymes will occasionally make errors. Because the pET vectors have low-copy, pBR322 origins of replication, plasmid yield from a standard miniprep is low. Typically, DNA from several minipreps is pooled or a larger-scale DNA preparation method is used to obtain sufficient DNA for sequencing. Alternatively, PCR with vector specific primers may be used to amplify and sequence the final insert.

3.2 Small-Scale Expression Trial

This section describes small-scale pilot expression studies (*see* succeeding chapters for larger scale expression and purification of the protein).

1. Transform BL21(DE3) and/or BL21(DE3)pLysS with the pET24d/chemokine construct. Also transform cells with empty vector as a negative control. Competent cells made using the TSS protocol ***(22)*** work well. Alternatively, competent cells may be purchased from Novagen.
2. For each construct, inoculate three 15-mL tubes with 5 mL LB containing 20 μg/mL kanamycin (and 10 μg/mL chloramphenicol, if using pLysS) with independent BL21(DE3) transformants. Also prepare a single sample with the pET24d transfomed cells. A master plate onto which each of the independent isolates has been patched should be made at this time. One frequently sees clone to clone variation in expression levels. This procedure allows selection of the highest expressing clone.
3. Grow at 37°C with vigorous agitation to stationary phase (8–16 h).

4. Inoculate a 15-mL tube with 5 mL LB containing 20 μg/mL kanamycin (and 10 μg/mL chloramphenicol, if using pLysS) with 50 μL of the stationary phase culture. Glycerol stocks should also be made from each of the overnight cultures by adding 300 μL sterile 80% glycerol to 700 μL overnight culture and freezing at –80°C.
5. Grow at 37°C with vigorous agitation to an OD_{600} of 0.4 (approx 2–3 h).
6. Add 5 μL of 1*M* IPTG (1 m*M* final concentration).
7. Grow at 37°C with vigorous agitation for 3 h.
8. Measure OD_{600} and transfer 0.4 OD_{600} units to a 1.5 mL microfuge tube.
9. Centrifuge at 12,000*g* for 2 min.
10. Discard supernatant and resuspend pellet in 100 μL 1X SDS sample buffer.
11. Heat to 95°C for 5 min.
12. Analyze 10 μL of sample by SDS-PAGE.

References

1. Makrides, S. C. (1996) Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol. Rev.* **60,** 512–538.
2. Weickert, M. J., Doherty, D. H., Best, E. A., and Olins, P. O. (1996) Optimization of heterologous protein production in *Escherichia coli*. *Curr. Opin. Biotechnol.* **7,** 494–499.
3. Studier, F. W. and Moffatt, B. A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189,** 113–130.
4. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorf, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185,** 60–89.
5. Dubendorff, J. W. and Studier, F. W. (1991) Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with *lac* repressor. *J. Mol. Biol.* **219,** 45–59.
6. Studier, F. W. (1991) Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *J. Mol. Biol.* **219,** 37–44.
7. Mertens, N., Remaut, E., and Fiers, W. (1995) Tight transcriptional control mechanism ensures stable high-level expression from T7 promoter-based expression plasmids. *Bio-Technology* **13,** 175–179.
8. Dong, H., Nilsson, L., and Kurland, C. G. (1995) Gratuitous overexpression of genes in *Escherichia coli* leads to growth inhibition and ribosome destruction. *J. Bacteriol.* **177,** 1497–1504.
9. Lopez, P. J., Lost, I., and Dreyfus, M. (1994) The use of a tRNA as a transcriptional reporter: the T7 late promoter is extremely efficient in *Escherichia coli* but its transcripts are poorly expressed. *Nucleic Acids Res.* **22,** 1186–1193.
10. Ramesh, V, Amitabha De, and Nagaraja, V. (1994) Engineering hyperexpression of bacteriophage Mu protein C by removal of secondary structure at the translation initiation region. *Prot. Engineer.* **7,** 1053–1057.
11. Kane, J. F. (1995) Effects of rare codon clusters on high-level expression of heterologous protein in *Escherichia coli*. *Curr. Opin. Biotechnol.* **6,** 494–500.

12. Chen, H., Pomery-Cloney, L., Bjerknes, M., Tam, J., and Jay, E. (1994) The influence of adenine-rich motifs in the 3' portion of the ribosome binding site on human IFN-γ gene expression in *Escherichia coli*. *J. Mol. Biol.* **240,** 20–27.
13. Proudfoot, A. E. I., Power, C. A., Hoogewerf, A. J., Montjovent, M.-O., Borlat, F., Offord, R. E., and Wells, T. N. C. (1996) Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist. *J. Biol. Chem.* **271,** 2599–2603.
14. Weber, M., Uguccioni, M., Baggiolini, M., Clark-Lewis, I., and Dahinden, C. A. (1996) Deletion of the NH_2-terminal residue converts monocyte chemotactic protein 1 from an activator of basophil mediator release to an eosinophil chemoattractant. *J. Exp. Med.* **183,** 681–685.
15. Gong, J.-H., Uguccioni, M., Dewald, B., Baggiolini, M., and Clark-Lewis, I. (1996) RANTES and MCP-3 antagonists bind multiple chemokine receptors. *J. Biol. Chem.* **271,** 10,521.
16. Campbell, E. M., Proudfoot, A. E. I., Yoshimura, T., Allet, B., Wells, T. N. C., White, A.-M., Westick, J., and Watson, M. L. (1997) Recombinant guinea pig and human RANTES activate macrophages but not eosinophils in the guinea pig. *J. Immunol.* **159,** 1482–1489.
17. Dong, H., Nilsson, L., and Kurland, C. G. (1996) Co-variation of tRNA abundance and codon usage in *Escherichia coli* at different growth rates. *J. Mol. Biol.* **260,** 649–663.
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Press, Cold Spring Harbor, NY.
19. Imai, T., Yoshida, T., Baba, M., Nishimura, M., Kakizaki, M., and Yoshie, O. (1996) Molecular cloning of a novel T cell-directed CC chemokine expressed in thymus by signal sequence trap using Epstein-Barr virus vector. *J. Biol. Chem.* **271,** 21,514–21,521.
20. Pan, Y., Lloyd, C., Zhou, H., Dolich, S., Deeds, J., Gonzalo, J.-A., Vath, J., Gosselin, M., Ma, J., Dussault, B., Woolf, E., Alperin, G., Culpepper, J., Gutierrez-Ramos, J. C., and Gearing, D. (1997) Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. *Nature* **387,** 611–617.
21. Dieffenbach, C. W. and Dveksler, G. S. (1995) *PCR Primer: A Laboratory Manual*. Cold Spring Harbor Press, Cold Spring Harbor, NY.
22. Chung, C. T., Niemela, S. L., and Miller, R. H. (1989) One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. USA* **86,** 2172–2175.

5

Expression of Chemokines in the Periplasmic Space of *E. coli*

Jochen Pfirstinger and Matthias Mack

1. Introduction

Here we describe the expression of chemokines in the periplasmic space of *Escherichia coli* ***(1–3)***, which leads to correctly folded proteins with no N-terminal modifications, in the case of RANTES, and with a yield of several hundred micrograms from a 1-L culture. Other expression systems that are generally used for the production of chemokines have several disadvantages. Bacterial expression in the cytoplasm of *E. coli* ***(4,5)*** gives large yields, but sometimes leads to an additional methionine at the N-terminus ***(6)***. Furthermore it requires an extensive refolding protocol, because disulfide bonds generally do not form in the cytoplasm, resulting in an incorrect protein conformation. Refolding is also necessary for chemically synthesized chemokines ***(7)***. Eucaryotic expression gives rise to correctly folded proteins, but has low yields, and secreted chemokines are often subject to proteolysis. In the case of RANTES expressed in CHO cells, a high proportion of the protein lacks the first two or three N-terminal amino acids.

The vector for protein expression in the periplasmic space of *E. coli* ***(3)*** (**Fig. 1**) contains a lac promotor, which can be induced with IPTG (Isopropyl-D-thiogalacto-pyranoside). Translation starts with the OmpA signal sequence of the "outer membrane protein A" of *E. coli*, followed by the chemokine cDNA. The signal sequence leads to protein secretion into the periplasm, where it is cleaved off by bacterial enzymes. The periplasmic space has an oxidizing milieu, which enables disulfide bond formation and contains molecular chaperones, which inhibit aggregation and support correct folding of proteins ***(8)***. The lysis of the periplasmic space is performed by four freeze/thaw cycles,

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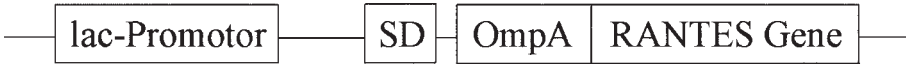

Fig. 1. Vector for protein expression in the periplasmic space of *E. coli*. Expression is controlled by an IPTG inducable lac promotor. SD is the Shine-Dalgarno sequence, OmpA is the signal sequence of the "outer membrane protein A" of *E. coli* followed by the RANTES cDNA.

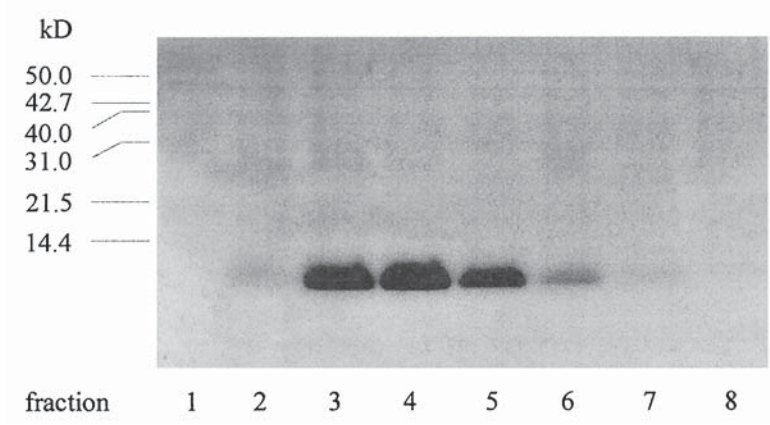

Fig. 2. Elution fractions of a purification of RANTES by affinity chromatography on Ni-NTA agarose visualized on a polyacrylamide protein gel.

resulting in a supernatant that is enriched in the chemokine and lacks most of the other bacterial proteins that are trapped in the spheroblast pellet.

As protease activity leads to a rapid degradation of chemokines, subsequent purification of the chemokine is advisable. If the chemokine carries an additional C-terminal 6xHis-tag, purification can be performed by single-step affinity chromatography with Ni-NTA agarose gel (QIAGEN GmbH, Germany). Elution is performed by a pH step gradient and several elution fractions containing the RANTES protein are visualized on a polyacrylamide protein gel (**Fig. 2**). The concentration of RANTES in the elution fractions was determined by enzyme-linked immunosorbent assay (ELISA) (**Fig. 3**). **Figure 4** shows a polyacrylamide protein gel loaded with periplasmic and eucaryotic expressed 6xHis-tagged RANTES, which was purified by affinity chromatography, compared to Met-RANTES, which was expressed in the cytoplasm of *E. coli* and purified from inclusion bodies.

Blocking of chemokine receptors by modifying their corresponding ligands is a promising therapeutic strategy in inflammatory diseases and HIV infection. With the method described here, chemokine derivatives can easily be expressed in a short time and in sufficient amounts.

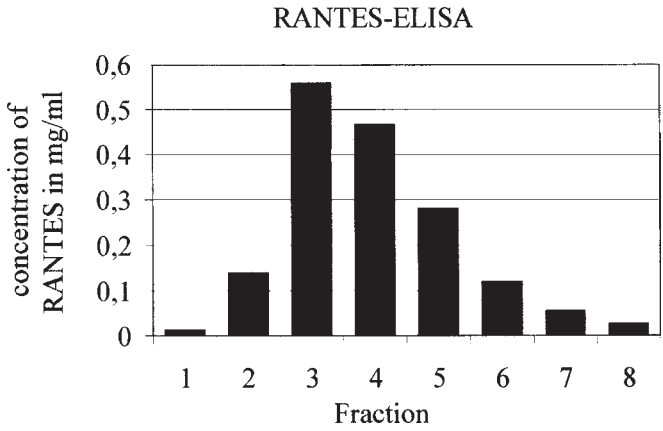

Fig. 3. Concentration of RANTES in the elution fractions shown in **Fig. 2** determined by ELISA.

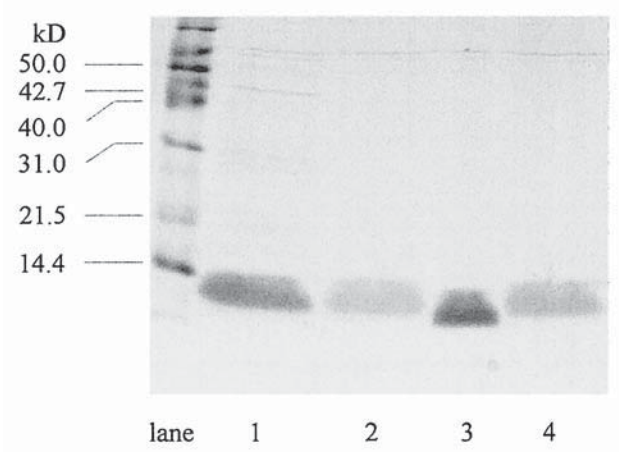

Fig. 4. Visualization of several purified RANTES proteins on a Coomassie stained SDS page: Lanes 1 and 2, 6xHis-tagged RANTES expressed in the periplasmic space of *E. coli*; Lane 3, Met-RANTES expressed in the cytoplasm of *E. coli* and purified from inclusion bodies; Lane 4, eucaryotic expressed 6xHis-tagged RANTES.

2. Materials

1. Expression vector as described in **Fig. 1** with chemokine cDNA insert.
2. *E. coli* strain JM83 for protein expression.
3. Ampicillin, 50 mg/mL in 70% ethanol.
4. Agar Petri dish.
5. LB medium.

6. 1 *M* IPTG stock solution dissolved in deionized water (Isopropyl-D-thiogalactopyranoside).
7. Sorvall centrifuge.
8. 15 mL COREX tubes.
9. Dry ice/ethanol bath and 37°C water bath.
10. QIAGEN Ni-NTA Agarose gel.
11. Pharmacia FPLC-device.
12. Loading buffer: 300 m*M* sodium chloride, 20 m*M* phosphate, pH 8.0.
13. Wash buffer: 300 m*M* sodium chloride, 20 m*M* phosphate, 10% glycerol, pH 6.5
14. Elution buffer A: 300 m*M* sodium chloride, 20 m*M* phosphate, pH 7.5
15. Elution buffer B: 300 m*M* sodium chloride, 20 m*M* phosphate, pH 3.0

3. Methods

1. Plate the transformed bacteria on an Agar Petri dish (with 100 g/mL Ampicillin) and incubate overnight at 37°C.
2. Transfer one colony into a flask with 50 mL LB medium containing 50 g/mL Ampicillin and shake overnight at 30°C.
3. Transfer 25 mL of the overnight culture into a flask with 1 Liter LB medium containing 50 g/mL Ampicillin and shake at 30°C until an optical density at 500 nm (OD_{500}) between 0.6 and 0.9 is reached (approx 3 h).
4. Induce the lac promotor with 1 mL 1 *M* IPTG (final concentration 1 m*M*) and shake the bacteria for 4 h at room temperature.
5. Cool down the bacteria on ice and centrifuge for 20 min at 2000*g* at 4°C.
6. Pour off the supernatant and resuspend the pellet completely in 20 mL 4°C cold loading buffer.
7. Fill the resuspended bacteria into six 15-mL glass tubes.
8. For lysis of the periplasmic space, resuspended bacteria have to undergo four freeze/thaw cycles as follows: put the COREX tubes alternately into a dry ice/ethanol bath and a 37°C water bath for 4 min each. After the last cycle, cool the tubes on ice.
9. Centrifuge the COREX tubes for 10 min at 16,000*g* at 4°C.
10. Recover the supernatant, which contains the proteins in the periplasmic space. Purification should be carried out immediately, or the solution frozen at –20°C, even for short-term use.
11. For purification, incubate the supernatant with 1 mL Ni-NTA agarose gel for 1 h at 4°C on a shaker.
12. Load the gel on a Pharmacia 10/10 column.
13. Wash with 40 mL wash buffer.
14. Elute the protein of interest by a pH step gradient, which can be generated by different proportions of elution buffers A and B according to the following protocol: 20 mL 100% buffer A and 0% buffer B; 20 mL 40% A and 60% B; 20 mL 20% A and 80% B (corresponding to pH 5.3); and 20 mL 0% A and 100% B. Collect the last 20 mL that contain the purified chemokine in 1 mL fractions.
15. Determine the purity of the chemokine with a polyacrylamide protein gel and the concentration by ELISA.

4. Notes

1. It is advisable to always keep the periplasmic lysate at 4°C, because protease activity leads to a rapid degradation of chemokines.
2. Lysis of the periplasmic space can alternatively be performed with BBS buffer (0.2 *M* sodium borate, pH 8.0, 0.16 *M* sodium chloride). This can lead to a lower yield and has the further disadvantage that dialysis may become necessary to eliminate the buffer. With BBS buffer proceed as follows:
   a. Resuspend the bacteria completely in 20 mL 4°C cold BBS.
   b. Shake the bacteria suspension for 30–60 min at 4°C.
   c. Centrifuge the bacteria as described in **Subheading 3., step 9**.
3. Binding of 6xHis-tagged proteins to Ni-NTA agarose gel is pH dependent. The best binding is achieved at a pH higher than 7.5.
4. In general, elution of 6xHis-tagged proteins does not take place at a pH higher than 4.5. However, elution conditions have to be optimized for every protein. If the protein is unstable at low pH, elution can also be performed with imidazole, a histidine analog.

References

1. Bird, R. E., Hardman, K. D., Jacobson, J. W., Johnson, S., Kaufman, B. M., Lee, S.-M., Lee, T., Pope, S. H., Riordan, G. S., and Whitlow, M. (1988) Single-chain antigen-binding proteins. *Science* **242,** 423.
2. Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margolies, M. N., Ridge, R. J., Bruccoleri, R. E., Haber, E., Crea, R., and Oppermann, H. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli. *Proc. Natl. Acad. Sci. USA* **85,** 5879.
3. Mack, M., Riethmüller, G., and Kufer, P. (1995) A small bispecific antibody construct expressed as a functional single-chain molecule with high tumor cell cytotoxicity. *Proc. Natl. Acad. Sci. USA* **92,** 7021.
4. Better, M., Chang, C. P., Robinson, R. R., and Horwitz, A. H. (1988) Escherichia coli secretion of an active chimeric antibody fragment. *Science* **240,** 1041.
5. Plückthun, A. and Skerra, A. (1989) Expression of functional antibody Fv and Fab fragments in Escherichia coli. *Meth. Enzymol.* **178,** 497.
6. Proudfoot, A. E. I., Power, C. A., Hoogewerf, A. J., Montjovent, M.-O., Borlat, F., Offord, R. E., and Wells, T. N. C. (1996) Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist. *J. Biol. Chem.* **271(5),** 2599.
7. Clark-Lewis, I., Moser, B., Walz, A., Baggiolini, M., Scott, G. J., and Aebersold, R. (1991) Chemical synthesis, purification, and characterization of two inflammatory proteins, neutrophil activating peptide I (interleukin-8) and neutrophil activating peptide 2. *Biochemistry* **30,** 3128.
8. Bardwell, J. C. A., Lee, J.-O., Jander, G., Martin, N., Belin, D., and Beckwith, J. (1993) A pathway for disulfide bond formation in vivo. *Proc. Natl. Acad. Sci. USA* **90,** 1038.

6

Synthesis of Chemokines

Ian Clark-Lewis

1. Introduction

Solid phase peptide synthesis (SPPS) is an alternative to DNA expression for generating proteins, such as chemokines ***(1–11)***. DNA databases and cDNA cloning has resulted in an explosion in the number of new chemokines from 1995–1998. However, for studies of the protein, knowing the DNA sequence is only the first step. The chemokine must be generated and in its correctly processed and folded form, and then purified to homogeneity. Expression of the cDNA is the popular route to the protein; however, *de novo* chemical synthesis has some significant advantages (e.g., efficient SPPS can provide 10–100 mg of pure chemokine in only a few days). Chemically synthesized chemokines have the same three-dimensional structures as ribosome-assembled chemokines made by recombinant DNA expression ***(3–5,7,9,10)***. Functionally, they are indistinguishable. The cumulative results of our chemokine studies have shown that chemical synthesis is a straightforward route to chemokines and their analogs. In this chapter, I describe the principles and procedures that colleagues and I have developed for synthesis of chemokines ***(1–11)***. Despite many advantages (*see* **Subheading 1.1., steps 1–10**), peptide synthesis has not been widely applied to proteins. One reason is that most researchers approach proteins from a biological, rather than a chemical, standpoint. Another is that in the early days of SPPS, the methods were limited to short peptides. However, gradual optimization of the chemistry eventually led to the synthesis of small proteins ***(12–14)***. Although the individual steps involved are straightforward, the methods require hands-on experience and a firm understanding of the principles involved.

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1.1. Advantages of the Peptide Synthesis Approach

1. Speed: A chemokine can be synthesized and purified in 10 days.
2. The methods for synthesis are straightforward, although the outcome of each step must be optimal for successful synthesis of proteins such as chemokines.
3. Amounts: Synthesis provides up to 100 mg of purified chemokine. The final yield that is obtained depends on many factors (*see* **Notes 5–9**). However, the amounts are usually sufficient to carry out in vitro studies, structure determination, and in vivo studies.
4. Purity: The primary structure of the synthetic chemokine product is defined by the sequence synthesized. Thus, there will be no variants due to adventitious processing or posttranslational modifications that can arise in material from recombinant sources.
5. N-terminus: For chemokines it is well-known that the N-terminal residues are critical for function ***(2–6,9)***. Sometimes the N-terminus of the mature protein is not known. Synthesis allows generation of multiple N-terminal forms in a single synthesis, thus allowing determination of the correct form.
6. Biological purity: As chemically synthesized products are totally nonbiological in origin, thus there is no chance of contamination with fMLP-related peptides, endotoxin or other biologically active molecules that could affect bioactivity.
7. Noncoded amino-acids: Chemical synthesis is not limited to coded amino acids and potentially allows the generation of an infinite variety of nonnatural analogs.
8. Cost: The initial cost of setting up a chemical synthesis laboratory is high, but once established, the cost of producing a chemokine by synthesis is at least competitive with DNA methods.
9. Scale: Synthesis of chemokines on an industrial scale is theoretically possible, but so far synthesis has been successfully applied on a scale that yields about 0.1 g of pure product. This is a major limitation of the synthesis method. Consistently obtaining high yields is a potential difficulty with large scale procedures. Cost also becomes factor. So far SPPS of proteins has been used as a research tool. Native ligation technologies ***(15)***, which will not be described here, have the potential to facilitate large scale protein synthesis
10. Equipment and expertise. Synthesis requires dedicated laboratory space and equipment. Protein synthesis is best done with the aid of a peptide synthesizer which is capable of optimal step-wise yields. Purification using reverse-phase high-performance liquid chromatography (RP-HPLC) is an integral part of the procedure ***(16)***, so at least one preparative and one analytical HPLC systems is needed. Access to electrospray mass spectrometry is essential.

1.2. Outline

In SPPS the C-terminal residue is covalently linked to a resin support, and appropriately protected amino acids are added one at a time until the complete polypeptide is formed. It is then deprotected and removed from the solid

support. The crude synthetic polypeptide is then folded and purified to homogeneity. *See* **Notes 1–4**, **10–14**, and **15–23** (**Fig. 1**).

2. Materials

2.1. Synthesis

1. The appropriate "pam" resin (tBoc-aminoacyl-(4-carboxyamidomethyl)-benzylester-poly-(styrene-1% divinylbenzene), where the "aminoacyl" group is the protected C-terminal amino acid corresponding to the C-terminal residue in the chemokine to be synthesized.
2. Protected amino acids that are used for the chain assembly are tBoc.Ala, tBoc.Asn(Xan), tBoc.Arg(Tos), tBoc.Asp(Bzl), tBoc.Cys(4MeBzl), tBoc.Glu(Bzl), tBoc.Gln, tBoc.Gly, tBoc.His(Bom), tBoc.Leu, tBoc.Ile, tBoc.Lys(2ClZ), tBoc.Met, tBoc.Pro, tBoc.Phe, tBoc.Ser(Bzl), tBoc.Tyr(2BrZ), tBoc.Thr(Bzl), tBoc.Trp(CHO) (light-sensitive), and tBoc.Val. Where tBoc = tertiary butyloxycarbonyl; Xan = xanthanyl; Tos = toluenesulfenyl; Bzl = benzyl; 4MeBzl = 4-methylbenzyl; Bom = benzyloxymethyl; 2ClZ = 2-chlorobenzoxycarbonyl; 2BrZ = 2-bromobenzoxycarbonyl; and CHO = formyl. Amino acids were obtained from Peptides International, Louisville, KY.
3. Dimethylformamide (DMF) and dichloromethane (DCM) (Burdick and Jackson, Muskeon, MI).
4. Trifluoroacetic acid (TFA) (Halocarbon, River Edge, NJ), and is redistilled before use.
5. Diisopropylethylamine (DIEA) (Perkin-Elmer-Applied Biosystems, Foster City, CA).
6. 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uroniumhexaflurophosphate (HBTU) (Richlieu, Montreal, QB).
7. Shaker model 504 (Peptides International).
8. Glass peptide synthesis reaction vessel (Peptides International).

2.2. Deprotection

1. Hydrogen fluoride 4 L tank (Matheson Gases Canada, Edmonton AB).
2. Cresol, thiocresol, dimethyl sulfide, Piperidine (Aldrich, Milwaukee, MI).
3. HF apparatus with all Teflon or Kel-F construction reaction vessels.
4. Calcium oxide trap (Peptides International, Louisville, KY).
5. Vacuum pump and KOH trap.
6. All Teflon 47mm filter holder (Savillex, Minnetonka, MN).
7. Zitex filter sheets no A115 (Chemplast, Wayne, NJ).
8. Shoulder length and standard neoprene gloves, and neoprene apron.
9. Face shield (Nalgene, Rochester NY).

2.3. Reverse-Phase-HPLC

1. RP-HPLC columns: C18 silica 300 Å pore size, 5 μm particle size, 250 × 4.6 mm; C18 silica 300 Å pore size, 5 μm particle size, 250 × 10 mm; C18 silica column

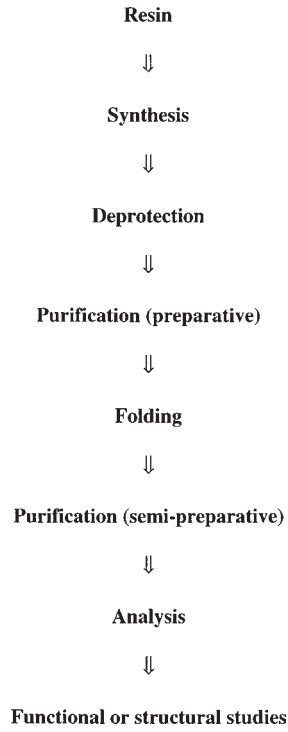

Fig 1. Steps in synthesis of chemokines.

300 Å pore size, 10–15 μm particle size, 22 × 250 mm (Vydac nos. 218TP54, 218TP510 218TP1022, respectively (The Separations Group, Hesperia, CA). RP-HPLC precolumns packed with C18-silica also Vydac.
2. Buffer A: 0.1% TFA in water (pass through a 0.2 μm filter; Millipore).
3. Buffer B: Acetonitrile containing 0.1% TFA (degas under vacuum).
4. Buffer C: 6 *M* guanidine HCl, 0.1 *M* Tris-HCl, pH 8.5.
5. 0.2 μm disposable filter 150 mL reservoir size; (Nalgene).
6. Analytical and semipreparative scale HPLC system capable of running binary gradients up to a maximal flow rate of 10 mL/min, UV detector, chart recorder and autosampler.
7. Preparative HPLC system capable of binary gradients and flow rates up to 50 mL/min, UV detector, and chart recorder.
8. Lyophilizer.

3. Methods

3.1. Chemokine Synthesis

This method is based on ***(17)***, and can be performed manually or with an automated synthesizer. The manual method is described below.

1. Add 0.5 mmol of resin (the resin amount corresponding to 0.5 mmol of the resin-bound amino acid) that had been prewashed with DCM, to a 40 mL fritted glass reaction vessel. Always add sufficient reagents to keep the resin in suspension, except during delivery or draining steps.
2. Remove the tBoc group by adding 20 mL 65% TFA/DCM for 1 min and drain, and then add a second aliquot of 65% TFA/DCM for 10 min.
3. Drain the reaction vessel and wash the resin thoroughly with DCM and then DMF, and neutralize the resin with 5% DIEA in DMF.
4. Form an active ester of amino acid (n-1) by addition of 2 mmol amino acid to 4 mL 0.45 *M* HBTU/DMF for 8 min, then add 1 mL DIEA for a further 2 min. The HBTU/DMF solution should be made fresh weekly.
5. Add the activated amino acid to the vessel and allow it to react for 15 min. Drain the vessel and then wash the resin with DMF.
6. Add a second 2 mmol of the same amino acid, that has been freshly activated by repeating **steps 4** and **5**. After 15 min, drain and rinse resin with DMF.
7. The resin now has residue n-1 linked to residue n which is attached to the resin.
8. Repeat **steps 2–6** for each amino acid until the entire protected polypeptide has been formed.

3.2. Cleavage and Deprotection

1. A modification ***(1)*** of the "low high" hydrogen fluoride (HF) method ***(18)*** is used. Compared to the simpler "high" only HF procedure, this method reduces modification of side chains, particularly cysteines.
2. The procedure is normally carried out using a Teflon apparatus, and care is taken to eliminate any likelihood of exposure to HF liquid or vapors. Note: HF is highly toxic.
3. Wash the resin with 20 mL DMF.
4. Add 5% piperidine in DMF to the resin and shake for 10 min at room temperature. Some chemokines contain no tryptophan, in which case this step can be eliminated.
5. Wash with DMF, and then DCM.
6. Remove the Nα tBoc group from the NH_2-terminal residue, using the deprotection and neutralization steps that have been described in **Subheading 3.1.**
7. Wash the resin with DCM 3 times, and then dry under vacuum.
8. For HF steps protective neoprene gloves, apron, and a plastic face shield should be worn. For the "low HF" step, add up to 4 g of resin to one of the Kel-F reaction vessels that are a part of the HF apparatus, and add 37.5 mL of a mixture of 90% dimethylsulfide, 5% p-thiocresol, 5% p-cresol and chill with liquid nitrogen and draw over 12.5 mL of anhydrous HF using vacuum created with a water driven aspirator to make a 25% HF solution. Remove liquid nitrogen.
9. After 1 h at 0°, carefully filter the peptide-resin from the mixture using a Teflon filter holder with a Teflon filter that was previously cut from a sheet of porous Teflon. Wash the resin with DCM. For the "high HF" step, add 5% p-thiocresol, 5% p-cresol, and 90% HF to the dried resin and allowed to react for 1 h at 0°C.

10. Draw off the HF into a 9*M* KOH trap using a gentle N_2 stream, and then draw off any remaining HF into a calcium oxide trap using a high vacuum pump. An oily mixture should remain.
11. This contains the polypeptide, resin, protecting groups, and the p-cresol/p-thiocresol.
12. To precipitate the peptide and extract the low molecular weight byproducts, add 20 mL ethylacetate and stir after 5 min, filter the precipitate through a Buchner funnel, and wash precipitate with more ethylacetate.
13. The precipitate contains the free, deprotected polypeptide and the original resin.
14. Wash the precipitate in 6*M* guanidine HCl, Tris 0.3 *M*, pH 8.5. The polypeptide will appear in the filtrate. Continue washing with sufficient 6*M* guanidine HCl, Tris 0.3 *M*, pH 8.5, and add 10% 2-mercaptoethanol, to make an approx 30 mg/ml solution.
15. Acidify this solution to pH 3.0 by addition of 20% acetic acid, store at –80°C.
16. This material is the crude synthetic product.

3.3. Analysis by RP-HPLC

1. Inject a small sample (e.g., 5 μL) on to an analytical RP-HPLC column which has been preequilibrated in buffer A.
2. Elute with a linear gradient up to 60% buffer B over 1 h. Record the UV absorbance at 214 nm, with the detector attenuation set at 2.0 absorbance units full scale.
3. Peptides have strong absorbance at this wavelength, and the loading should be adjusted so that the maximum peptide peak is 50 to 75% of full scale.
4. The molecules do not elute as discrete peaks but rather coalesce to give a broad chromatogram with a major peak.
5. Chromatograms of crude product of a chemokine synthesis is shown in **Fig. 2A**.
6. Retain this chromatogram for future reference as it provides the profile of the original crude synthetic product and can be used to compare syntheses.

3.4. Folding

1. Establish folding conditions by preparing a series of 0.5 mL, 1 mg/mL samples of the chemokine polypeptide, using the solvents and conditions listed here.
   a. 1 *M* guanidine HCl, 0.1 *M* tris, pH 8.5, 10% DMSO in a sealed tube, in the dark without stirring.
   b. 1 *M* guanidine HCl, 0.1 *M* tris pH 8.5, stir in air.
   c. Water, stir vigorously in air (avoid frothing).
   d. 10% DMSO in a sealed flask, in the dark without stirring.
   e. PBS, stir in air.
2. After 24 h, analyze each sample by RP-HPLC.
3. Assess folding by comparing the chromatogram with that of the unfolded sample and look for conversion to a peak that elutes about 3 min earlier than the unfolded, based on a 1%/min gradient. The extent of conversion to this early

eluting form reflects the extent of folding. Select the conditions that give optimal conversion for folding of the remaining material as a single large batch. (*See* **Notes 10–14.**)

3.5. Purification

The following strategy is designed for the crude product from an entire synthesis, i.e., about 2 g of product ***(1,16)***. (*See* **Notes 5–14.**)

1. Crude product (**Fig. 2A**).
2. Preparative RP-HPLC (**Fig. 2B**).
3. Folding (**Fig. 2C**).
4. The semipreparative scale RP-HPLC (**Fig. 2D**).
5. Repeat of **step 4**, if necessary.

3.5.1. Preparative RP-HPLC

1. Load the crude synthetic product obtained from the cleavage step directly on to a preparative column through the solvent inlet of the "A" pump.
2. Apply a 200 min A to B solvent gradient at 15 mL/min and monitor protein elution at 214 n*M*.
3. Collect fractions every 1.0 min, and run 20 μL each on the analytical column as described in **Subheading 3.3.**
4. Identify the fractions containing the major peak by comparison with the chromatogram of the crude product. Pool the selected fractions and lyophilize.

3.5.2. Semipreparative RP-HPLC

A similar procedure is used for semipreparative RP-HPLC except the amount loaded is 50 to 100 mg of protein.

1. Run a gradient of 0–60% acetonitrile over 200 min at a flow rate of 3 mL/min and collect fractions every 1.5 min.
2. Run fractions on analytical RP-HPLC as described in **Subheading 3.3.**
3. Pool and lyophilize the fractions that contain a single peak.

3.6. Mass Spectrometry

1. Prepare a 1 mg/mL solution (~100 μL) of purified chemokine in 2% acetonitrile 0.5% TFA.
2. Inject the sample at a rate of 5 μL/min using a Harvard pump into the injection port of a precalibrated electrospray mass spectrometer.
3. Collect charge/mass signals from the ionized sample for 1 min.
4. Analyze the data using the HYPERMASS program to generate the average measured mass, and the standard deviation of the mass measurement.
5. Compare the measured mass obtained with the mass calculated using the promass program. When calculating the mass, don't forget to take into account the disul-

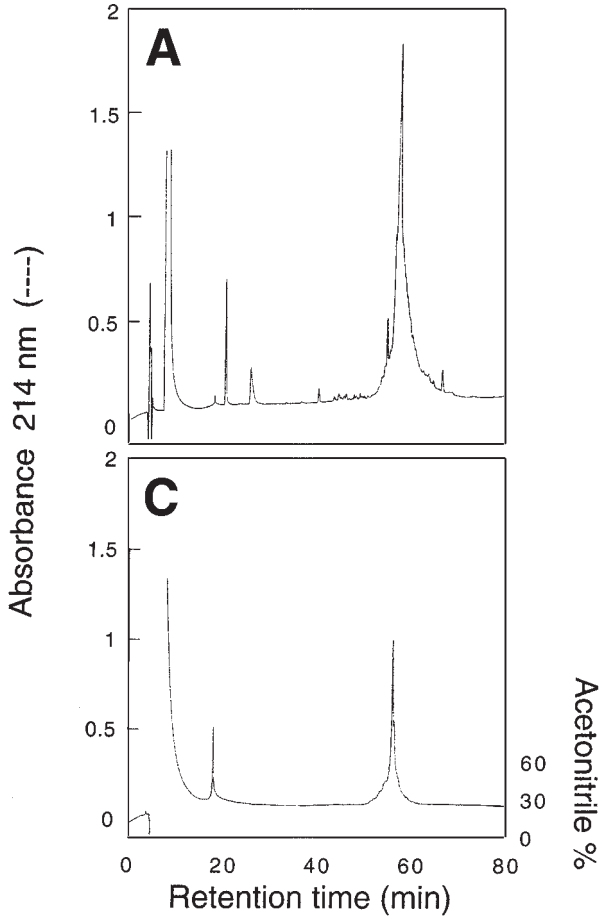

Fig. 2. RP-HPLC chromatograms of human B cell chemoattractant-1 (BCA-1) ***(11)***, an 87 residue chemokine that is the ligand for CXCR5, which is the human homolog of the mouse receptor BLR-1. Its sequence is: VLEVYYTSLRCRCVQESSVFIPRR FIDRIQILPRGNGCPRKEIIVWKKNKSIVCVDPQAEWIQRMMEVLR KRSSSTLPVPVFK ***(11)***. The BCA-1 polypeptide was synthesized starting with Lys-Pam resin and adding the appropriate protected amino acids until the N-terminal valine was added. The profile of the crude product **(A)** reveals a major broad peak that consists of the correct polypeptide and numerous closely related and closely eluting

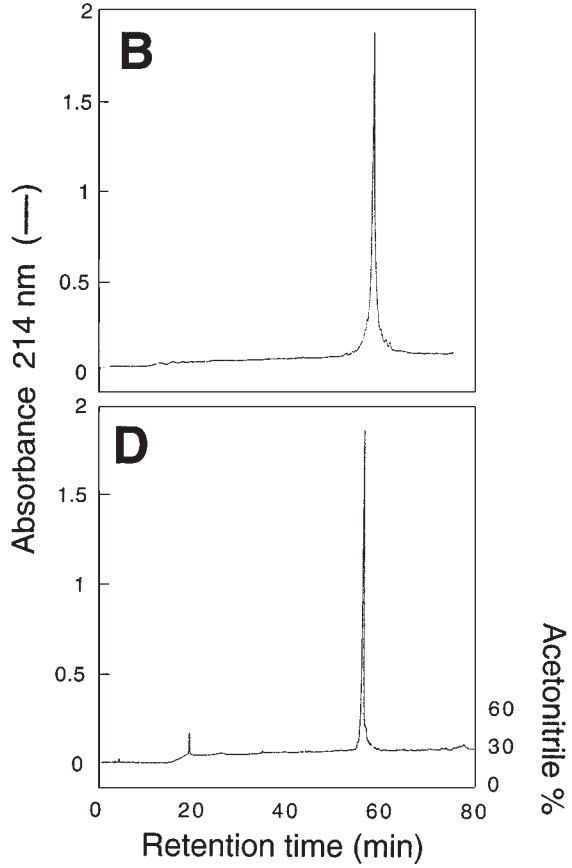

Fig. 2. *(continued)* contaminants, which are generally not resolved, although some are apparent as shoulders on the main peak. The crude product was run on preparative RP-HPLC and selected fractions were pooled and lyophilized and a sample run on analytical RP-HPLC **(B)**. The peak is significantly sharper as much of the contaminating material has been eliminated. This material was folded in 10% DMSO and an analytical RP-HPLC profile is shown in **C**. The retention time of the major folded peak is about 2 mm or 3% earlier than the unfolded material. The difference is easily seen bycomparing A and C or B and **D**. After a final RP-HPLC purification step selected fractions were pooled and the resulting final product is shown in D.

fides mass. If the sample is pure, peaks corresponding to only one molecular species should be apparent.

See **Notes 26–28.**

3.7. Storage

1. After purification store the lyophilized chemokine at –80°C.
2. For use, prepare a stock solution by dissolving a carefully weighed sample of chemokine in water to make a 10 mg/mL solution and store aliquots at –80°C. This can be diluted into medium or buffer, as required.

4. Notes

4.1. Synthesis

1. Synthesis of the protected polypeptide always starting with the carboxyl-terminal amino acid and proceeds with the addition of amino acids one at a time, and ends with the addition of the amino terminal amino acid. The synthesis steps can be performed manually using a fritted glass reaction vessel with a Teflon valve, however, it is more efficient to use a programmable peptide synthesizer, which is capable of performing the steps faster and more reproducibly, than can be accomplished manually.
2. tBoc vs Fmoc. Two different strategies are used for SPPS. They are designated tBoc and Fmoc, according to the abbreviation for α-amino protecting group. In the tBoc strategy the α-amino groups of all the amino acids are protected with tBoc; likewise for Fmoc, they are all Fmoc protected. The chemistry of the side chain protecting groups differs in the two strategies. The most widely touted advantage of Fmoc synthesis is that it does not require hydrogen fluoride for deprotection. The milder deprotection method that is used is simpler and safer. Compared to the Fmoc method, tBoc synthesis can be expected to provide higher overall yield. Nevertheless, either tBoc or Fmoc method could potentially be used for synthesis of chemokines. In my laboratory we have focused on the tBoc method, which we have found to be reliable and has given excellent yields.
3. Length. Most chemokines are about 70–80 amino acids in length, although this is not universal as some are considerably larger, for example, TECK has 127 residues ***(19)***. Polypeptide length is important in synthesis because there is an exponential increase in number of byproducts and concomitant reduction of peptide yield as the length increases. The quality of the final product is dependent on the sequence as well as the length. The chemistry can never be 100% efficient and eventually a size limitation is reached. Nevertheless, the length limit of the methods described here is within the length of most chemokines. Newer methods that involve linking of synthetic fragments together to form the complete protein potentially extends the capability of chemical synthesis ***(15)***. These methods are not yet widely used and will not be discussed here.
4. Resin splitting. Splitting the resin at intermediate stages allows synthesis of several different peptides with a common C-terminus. This procedure is particularly

useful for chemokines as most of the functional motifs are near the N-terminal region. The resin can be split up to 10 different ways and the intermediate peptide-resin stored at –80°C in DMF, and the synthesis continued at another time. Avoid drying the peptide-resin. As expected, splitting lowers the yield of each product, and in our experience 10 splits is the maximum.

4.2. Yields

5. Two distinct types of yield are important in SPPS. First, the step-wise yields, which reflects the efficiency and fidelity of the chemistry at each residue in the synthesis, and second, the yields in folding and purification.
6. Step-wise yields. The most notorious byproducts are shortened by one or more residue and result from incomplete reaction of the amino acid to the peptide (coupling step) and lead to byproducts which have one or more residues missing called deletion products. These deletion byproducts are closely related to the final product and therefore can be difficult to resolve. This difficulty has been counteracted by improvements to the chemistry that allow coupling yields of average 99.5%, and thereby reduce these byproducts to a manageable level. For example, a second coupling step with a second aliquot of activated amino acid, improves the average step-wise yield by 0.3–0.5% and thus significantly increases final overall yield. (Note: that the second coupling does not result in double addition because the tBoc protecting group prevents reactivity.) However, the yields are unlikely to reach 100%. The deletion products are not the only byproducts of the chemistry that reduce stepwise yields. Others arise from impurities in the amino acids, and various chemical modifications and side reactions that occur during either synthesis or deprotection. These are highly sequence dependent and not readily quantifiable.
7. The overall synthetic yield is obtained by: % Synthetic yield = (average step-wise yield)^n × 100. Where n is the number of residues, and the average step-wise yield expressed as a fraction. This relationship illustrates that the final yield always decreases exponentially as the length increases. Furthermore, it illustrates the corollary that the number of byproducts also increases as a function of chain length. Consequentially not only are yields lower, but it is more difficult to obtain pure product from longer syntheses.
8. In practical terms, the overall yield is affected by length because the resin swells as the peptide grows, but the maximum amount of peptide-resin that can be accommodated in a standard 40 mL reaction vessel is about 4 g. Thus, irrespective of other factors the overall yield of crude peptide is limited to about 2 g (50% of the weight of the peptide resin is resin and protecting groups). In principle the yield can be increased by increasing the size of the reaction vessel instead of removing resin, but the concentration of reagents must be maintained so more reagents, including amino acid, will be required.
9. Final yield. The final yield is the amount of pure product obtained. It is determined by the synthetic yield factors above and also on the recovery during purification on RP-HPLC. If the product cannot be identified in the crude prod-

uct or is not clearly apparent after folding steps, then it is questionable whether the protein can be isolated as a pure product.

A general observation is that the more time that it takes to complete a synthesis, the lower the quality of the final product. The exact basis for this unknown, but it is possible that incipient side reactions occur even when the peptide-resin is standing. Thus, the synthesis is best done rapidly and continuously.

4.3. Folding

10. In the case of chemokines a stable "folded" protein is achieved when the dissulfide bridges form. Under optimal conditions when the secondary and tertiary structure form, the disulfides also form.
11. Folding. Chemokines, like most globular proteins, exist in a compact folded form that is unique to its sequence. In the folding process the tertiary structure is adopted spontaneously. Secondary structure forms and nonpolar side chains associate to hydrophobic core. Finally the two disulfide bridges are formed, after which the molecule is quite stable to denaturation and chromatographic methods. Thus, in the in vitro folding process it is essential that optimal conditions be used for formation of the tertiary structure and allow the disulfides to form. Importantly the polypeptide must be soluble in **both** the unfolded and folded form. Precipitation or aggregation in the folding reaction will dramatically affect yields.
12. The folding process results in a compact structure with a proportion of the nonpolar side chains buried. This means that, relative to the unfolded form, the folded form has a more polar surface accessible area. This difference is apparent by RP-HPLC, and thus the extent of folding can be readily evaluated by RP-HPLC. The folded material characteristically elutes 1–3 min earlier than the unfolded material. Later eluting peaks contain material that either cannot fold, or did not fold. Since folding separates the material that readily folds from the various byproducts that cannot fold, the folding process actually assists in the purification of the final chemokine product.
13. On RP-HPLC this may be apparent as a late eluting broad rounded lump. Therefore it is best to check a number of different conditions on a small scale before committing the entire product to a particular method. Solvent variables include the denaturant concentration guanidine HCl, pH, buffers, ionic strength, etc. It is best to avoid using urea as a denaturant because of the possibility of protein modification.
14. The second requirement is the addition of a mild oxidant that will convert the SH group to S^-, but not to higher oxidation states. It is essential that the oxidation of the SH group be reversible. Examples of suitable oxidants are DMSO or oxygen, which is kept dissolved by vigorous stirring.

4.4. RP-HPLC Analysis and Purification

15. Purification of synthetic proteins requires approaches that separates closely related byproducts from the correct molecule. Affinity chromatography for example, is not useful for synthetic peptides. Routinely used are chromatographic

techniques that separate on the basis of physicochemical properties such as the stability, solubility and polarity of the solvent-accessible surface. These factors are determined by the amino acid sequence and/or the folded structure. Most of the byproducts are closely related, but different from, the polypeptide of interest and by RP-HPLC they give the appearance of being unresolved as they coalesce to give the initial crude product broad lumpy appearance (**Fig 2A**).

16. RP-HPLC and peptide/protein synthesis are compatible methods because peptides are soluble in RP-HPLC solvents and interact reversibly with the hydrophobic matrix. In addition the peptide and synthetic byproducts are well resolved. Thus, RP-HPLC is ideal for analysis of the crude synthetic product, as well as monitoring the various stages of folding and purification (**Fig 2**). A major advantage of RP-HPLC is that it can be used over a wide range of scales, e.g., for analysis and purification. Resolution of the method is largely retained over a wide range of column sizes with differing capacities. The purification progress is monitored by comparison of analytical RP-HPLC chromatograms starting with the crude product and generated progressively during the purification.
17. For analysis by RP-HPLC it is necessary to record the chromatogram at 40–80% of the maximal setting. 2.0 AUFS is preferable as this eliminates drift due to UV absorbance of acetonitrile and also baseline noise. Running the analytical chromatograms under exactly the same conditions facilitates comparisons of the protein at different stages of the purification. Peptides have a strong UV absorbance band around at 214 nm due to the peptide bond, and it is essential that this wavelength be monitored otherwise the results will be distorted and unreliable. Modified synthetic byproducts often have skewed spectral characteristics and can be detected by, dual or multi-wavelength monitoring at higher UV wavelengths, e.g., 280 nm.
18. Low loading on to preparative columns results in poor yields due to nonspecific losses. On the other hand, overloading could cause peak broadening due to solubility problems on elution from the column. RP-HPLC columns have high capacities for protein retention and a loading of about 50% of total capacity of the column is best. A guide is: 2 g for 25 × 250 mm column, 100 mg for (10 × 250 mm column) and 5 mg for (4.6 × 250 mm column).
19. It is difficult to directly compare the preparative chromatogram with the analytical profile because of the high absorption of the peptide. This could be overcome with HPLC systems that have stream splitting. Although cumbersome, the best way to select the fractions is by running them on analytical scale RP-HPLC and directly comparing the profiles with the analytical profile of the original sample.
20. It is essential that pooled fractions be lyophilized after each RP-HPLC step. This is because the protein is denatured in water-acetonitrile-TFA, so to obtain a clean, unimolecular interaction of the polypeptide with the column there should be no aggregation. The sample is loaded in aqueous solution.
21. Doing the first RP-HPLC purification step before folding eliminates contaminants that interfere with folding, including hydrophobic byproducts that could cause precipitation, and reducing agents. Thus, it avoids the need for a Sephadex

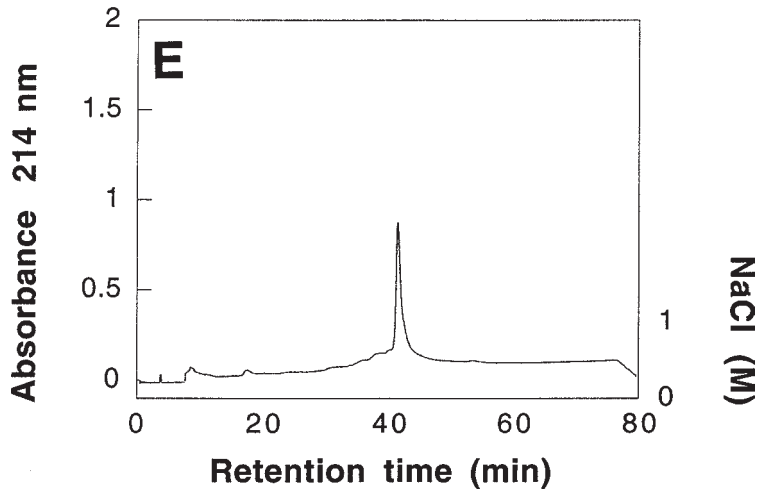

Fig. 2. *(continued)* Panel **E** shows the chromatogram when the BCA-1 in D was run on analytical ion-exchange (sulfonic acid derivatized HPLC column). The gradient is 0 to 1 *M* sodium chloride in 0.05*M* K2P04 pH 6.0 and 10% acetonitrile. As is typical the peak is not as sharp as with RP-HPLC but nevertheless only one peak is apparent suggesting that the material in D is pure.

G25 step to exchange the crude material into folding buffer, as would be necessary if the folding was to be done first.

22. With difficult samples, for which the peaks cannot be resolved by RP-RP-HPLC, an alternative purification method such as preparative cation-exchange HPLC on a sulphonate derivatized column could be used.
23. The lyophilized samples from RP-HPLC contain TFA salts of the peptide. Although this is not a problem between RP-HPLC steps, it could be important the final pure product. The TFA will exchange slowly in buffered solutions but may interfere with some methods, such as crystallization. TFA salts and residual solvents can be removed by addition of 0.01*M* sodium hydroxide, and then exchanging the protein on a Sephadex G-25 column equilibrated with water and lyophilizing.

4.5. Evaluation of Purity

24. Purity is indicated by a clean, single, sharp peak on analytical RP-HPLC. The presence minor peaks of shoulders or a distorted peak shape, are indicators that the material needs further purification. To further confirm purity an orthogonal separation method should be used. For example, analytical ion-exchange HPLC, **Fig 2E**, for which the separation is based on ionic rather than nonpolar interactions. Analytical isoelectric focusing can also be used.

25. For most chemokines RP-HPLC is the only purification technique needed. However use of only one purification method may not always result in a pure product. In some cases another separation method such as ion-exchange HPLC is needed to obtain purity. This usually resolves contaminants that were not resolved by RP-HPLC.

4.6. ESMS: Verification of Covalent Structure

26. It is essential to verify that the purified material has the covalent structure that was originally intended. A complete covalent structure determination, including sequence determination and chemical composition analysis, and the disulfide bridges, is neither practical nor necessary. In any event these analytical techniques have their limitations as they may not identify certain covalently modified byproducts.
27. From a practical perspective electrospray mass spectrometry (ESMS) provides an accurate measures of the molecular mass ***(1,2,19)***. All chemokines tested have given multiple charged species from which the mass is calculated. The structure can be inferred from the mass with high probability and therefore it is the most powerful method available for the analysis of synthetic peptides and proteins. If the measured mass is the same as the calculated mass and reasonable care has been taken in the synthesis (i.e., no mistakes such as Leu to Ile), ESMS provides strong evidence (but does not prove) that the correct molecule has been made. On the other hand, if the mass detected is incorrect then there is no doubt that wrong molecule has been synthesized and/or isolated. A major advantage of this mass spectrometric method is that the whole protein is analyzed without fragmentation or chemical treatment. This is important because analysis of fragments does not necessarily imply that the full-length biologically active molecule is correct. The electrospray method has the advantage of speed, high accuracy (for a chemokine with a mass of 8, 000 ± 1 amu), and reliability. RP-HPLC solvents are compatible with the analysis so the protein can be easily evaluated during purification. It should be noted however that some amino acid replacements could be within 1 amu and therefore not distinguishable. ESMS plus technical proficiency in forming the correct sequence, equals verification of the covalent structure.
28. Formation of two disulfides results in a mass 4 amu lower than the reduced form and this is readily detectable by ESMS. This indicates that the two disulfides have formed. It is most likely that the pairing is correct but the analytical method does not prove that the disulfide pairing is correct. To do this it is necessary to fragment the molecules and isolate each disulfide on two different fragments. This is difficult with chemokines because the disulfide pairs are extremely close. Nevertheless, NMR structures of synthetic chemokines and their analogs, show that cysteine pairing is correct ***(3,4,7,9,10)***. It seems that the 1 to 3 and 2 to 4 pairs are favored during the folding process. Thus, mass spectroscopy is adequate for verification of disulfide formation but is not absolute proof of the disulfide pairing or of structure.

Acknowledgments

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References

1. Clark-Lewis, I., Moser, B., Walz, A., Baggiolini, M., Scott, G. J., and Aebersold, R. A. (1991) Chemical synthesis, purification and characterization of two inflammatory proteins, neutrophil activating peptide-1 (interleukin-8), and neutrophil activating peptide-2. *Biochemistry* **30,** 3128–3135.
2. Clark-Lewis, I., Dewald, B., Loetscher, M., Moser, B., and Baggiolini, M. (1994) Structural requirements for IL-8 function identified by design of analogs and CXC chemokine hybrids. *J. Biol. Chem.* **269,** 16,075–16,081.
3. Rajarathnam, K., Clark-Lewis, I., and Sykes, B. D. (1994) ^{1}H NMR studies of interleukin-8 analogs: characterization of the domains essential for function. *Biochemistry* **33,** 6623–6630.
4. Kim, K.-S., Clark-Lewis, I., and Sykes, B. D. (1994) Solution structure of GRO/MGSA determined by ^{1}H NMR spectroscopy. *J. Biol. Chem.* **269,** 32,909–32,915.
5. Clark-Lewis, I., Kim, K.-S., Rajarathnam, K., Gong, J.-H., Dewald, B., Baggiolini, M., and Sykes, B. D. (1995) Structure-activity relationships of chemokines. *J. Leukocyte Biol.* **57,** 703–711.
6. Gong, J.-H. and Clark-Lewis, I. (1995) Antagonists of monocyte chemoattractant protein-1 identified by modification of functionally critical NH_2-terminal residues. *J. Exp. Med.* **181,** 631–640.
7. Kim, K.-S., Rajarathnam, K., Clark-Lewis, I., and Sykes, B. D. (1996) Structural characterization of a monomeric chemokine: monocyte chemoattractant protein-3.*FEBS Lett.* **395,** 277–282.
8. Gong, J.-H., Uguccioni, M., Dewald, B., Baggiolini, M., and Clark-Lewis, I. (1996) RANTES and MCP-3 antagonists bind multiple chemokine receptors. *J. Biol. Chem.* **271,** 10,521–10,527.
9. Crump, M. P., Gong J.-H., Loetscher, P., Rajarathnam, K., Amara, A., Arenzana-Seisdedos, F., Virelizier, J.-L., Baggiolini, M., Sykes, B. D., and Clark-Lewis, I. (1997) Solution structure and basis for functional activity of stromal cell-derived factor-1: dissociation of CXCR4 activation from binding and inhibition of HIV-1. *EMBO J.* **16,** 6996–7007.
10. Crump, M. P., Rajarathnam, K., Kim, K.-S., Clark-Lewis, I., and Sykes, B. D. Solution structure of the chemokine eotaxin: an eosinophil specific chemoattractant. *J. Biol. Chem.* in press.
11. Leger, D. F., Loetscher, M., Stuber-Roos, R., Clark-Lewis, I., Baggiolini, M., and Moser, B. M. (1998) BCA-1, a human CXC chemokine expressed in lymphoid

tissues, selectively attracts B lymphocytes via BLR1/CXCR5 *J. Exp. Med.* **187,** 655–660.
12. Clark-Lewis, I., Aebersold, R. A., Ziltener, H., Schrader, J. W., Hood, L. E., and Kent, S. B. H. (1986) Automated chemical synthesis of a protein growth factor for hemopoietic cells, interleukin-3. *Science* **231,** 134–139.
13. Schneider, J. and Kent, S. B. H. (1988) Enzymatic activity of a synthetic 99 residue protein corresponding to the putative HIV-1 protease. *Cell* **54,** 363–368.
14. Kent, S. B. H. (1988) Chemical synthesis of peptides and proteins. *Ann. Rev. Biochem.* **57,** 957–989.
15. Clark-Lewis, I. and Kent, S. B. H. (1989) The use of HPLC in protein purification and characterization, in *Receptor Biochemistry and Methodology* (Ventor, J. C. and Harrison, L. C., eds.) A. R. Liss, New York. NY, pp. 43–75.
16. Dawson, P. E., Muir, T. W., Clark-Lewis, I., and Kent, S. B. H. (1994) Synthesis of proteins by native chemical ligation. *Science* **266,** 776–779.
17. Schnolzer, M., Alewood, P., Jones, A., Alewood, D., and Kent, S. B. H. (1992) In situ neutralization in Boc-chemistry solid phase peptide synthesis. *Int. J. Pept. Prot. Res.* **40,** 180–193.
18. Tam, J. P., Heath, W. F., and Merrifield, R. B. (1983) SN2 deprotection of synthetic peptides with a low concentration of HF in dimethyl sulfide: evidence for application in peptide synthesis. *J. Am. Chem. Soc.* **105,** 6442–6455.
19. Chait, B. T. and Kent, S. B. (1992) Weighing naked proteins: practical, high-accuracy mass measurement of peptides and proteins. *Science* **257,** 1885–1894.

7

Identification of Novel Chemokines From Expressed Sequence Tag Databases

Timothy N. C. Wells and Manuel C. Peitsch

1. Introduction

The chemokine superfamily is a large group of small proteins, which share a limited amount of primary sequence identity, although a highly conserved three-dimensional subunit structure. The original members of the superfamily, Interleukin-8 and MCP-1 were purified over 10 years ago. Following on from these discoveries, many more of these proteins were identified because of their ability to selectively recruit and activate specific leukocyte populations. From these initial discoveries, the number of chemokines that have been discovered has increased almost exponentially in the last two years. Two main factors have been key to the discovery of so many new chemokines: first, the availability of large amounts of sequence information gained by random sequencing of cDNA libraries generating expressed sequence tag collections (ESTs); and second, the availability of this information on-line to research groups via the world wide web. This has meant the rapid increase in the number of chemokines that are currently well characterized. An additional spin-off of these novel chemokines is that a number of putative chemokine receptors have been paired up with their ligands over the last year.

The discovery of novel chemokines essentially occurred in three waves. First of all, many chemokines were purified from cell culture media, or leukocyte exudates. These were the first chemokines to be identified, and include the two mainstays of chemokine biology, Interleukin-8 (IL-8) and Monocyte Chemoattractant Protein-1 (MCP-1). It is interesting that chemokines could indeed be purified in this way, compared with other cytokines, which have tended to be identified initially as cDNAs. One of the reasons that chemokines

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could be identified and sequenced, is that they are very stable proteins, and are sufficiently small that unlike other proteins they can be purified on HPLC. Another reason for their identification, is that they tend to show activities in the low nanomolar range, and therefore are produced at high concentrations. Other cytokines are quite often active in the subpicomolar range, and so are produced at lower levels which means that it is effectively impossible to purify and sequence the proteins.

The second wave of chemokines were those identified by cDNA cloning, or by polymerase chain reaction (PCR) using sequence motifs that are well conserved between chemokines. It includes members such as RANTES, which were identified because of changes in expression levels during the inflammatory response. Identification of such novel chemokines is made easy not only because of the characteristic cysteine motif which is present at the amino terminus of the proteins, but also because of a number of other sequence similarities which occur. These similarities have given us guidelines which have enabled us to identify novel potential chemokine open reading frames. All chemokines identified to date have an amino terminal cysteine motif where the first two cysteines are adjacent (as in MCP-1), separated by one amino acid (as in Interleukin-8), separated by three amino acids (as in Fractalkine/ Neurotactin) or where there is only one cysteine (as in Lymphotactin). With the exception of Lymphotactin, all chemokines have at least four cysteine residues, whose position in the sequence makes chemokine reading frames easy to identify. In addition to the cysteine motifs, there are other highly conserved sequences. Towards the end of the initial 70 amino acid region there is invariably a carboxy terminal helix starting about 7 amino acids from the fourth conserved cysteine residue. Even without carrying out three-dimensional modeling procedures, this helix can be seen from the way that the hydrophobic residues (often starting with a tryptophan and valine pair) can be seen to occur on the 1st, 4th, 5th, 8th, and 11th residue, the classical helical wheel. (**V**QR**VV**EK**F**KK**R** for IL-8). In addition, the similarities in the region of the second and third beta sheet can often be identified. The basis of these rules becomes much clearer when the three-dimensional structures are compared (*see* **Fig. 1**). More recently the cDNA cloning approach has been extended to Signal Sequence Trap methods which are aimed at selectively isolating 5' cDNA fragments carrying signal sequences. This was the approach used to identify Stromal Derived Factor-1 (SDF-1) ***(1)***, and also TARC, one of the ligands for CCR4 ***(2)***. It should be noted that in the case of SDF the authors were not looking specifically for chemokines, but the Signal Sequence Trap method has been a very fruitful source of new cytokines.

The third wave of chemokines came from the use of EST databases. Many groups have been obtaining large amounts of sequence data simply by taking a

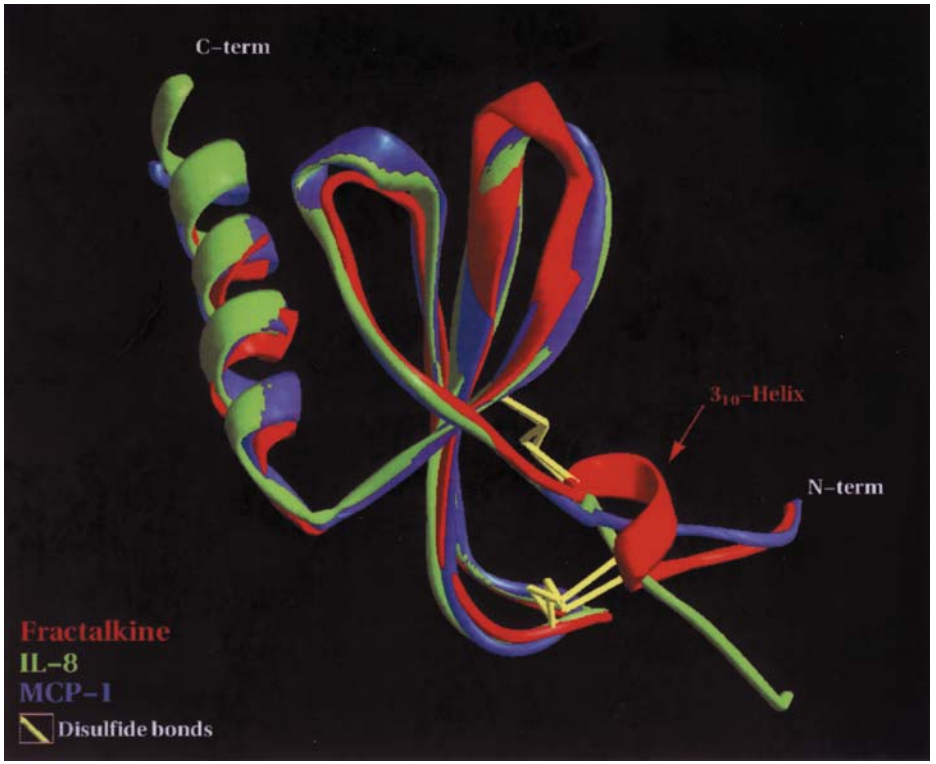

Fig. 1. Comparison of the three-dimensional structures of human Interleukin-8 (green) MCP-1 (blue) and Fractalkine (EST Z44443) (red). The Il-8 structure is taken from the Protein Database (PDB) entry (1IL8), and the MCP-1 structure is a model built of the NMR structure of MIP-1β (PDB entry 1HUM). The intrachain disulfide bonds are shown in yellow. The model for the chemokine domain of Fractalkine was built using the SwissModel server ***(16,17)***. As can be seen the three structures show a large degree of conservation of the overall structure, despite a relatively low level of primary sequence identity. The additional three amino acids in Fractalkine are accommodated as a 3_{10} helix between the two N-terminal cysteines. The steric requirements here presumably forbid a CX_2C motif. The model building software can be accessed at http:\\www.expasy.ch\swissmod\SWISS-MODEL.html

cDNA library from a tissue or cell type of interest, and sequencing as many clones as possible, generating ESTs. The idea is that the 250–500 bases of DNA sequence that is obtained gives a tag system which enables us to catalog all of the genes which are expressed in the human genome. In the case of chemokines, where a typical open reading frame is only coding for 100 amino acids, there is an added advantage, in that the entire coding region for a chemokine ligand

is likely to be found on a single EST. Given that the rules on how to identify new chemokines are clear from the primary peptide sequence, it was relatively easy to find new members of the superfamily. There are now over a million EST sequences available, on public domain databases, meaning that it is often possible to find several examples of the same open reading frame, allowing us to correct elementary sequencing errors even before starting to clone to cDNA itself.

2. Methods

2.1. Finding Sequences

The use of EST databases to identify novel chemokines, only using computers, has been very fruitful (*see*, for example, **refs. *3,4***). The basic method involved setting up TBlastN searches for chemokines using a variety of starting chemokines: MIP-1α, IL-8, MCP-1, etc. (the NCBI home blast page can be found at http:\\www.ncbi.nlm.nih.gov\). This searches the EST database for similarities to known chemokines in the three forward and three reverse reading frame. In this way the selection is biased towards the amino acid identity. Once initial hits had been identified then automated searches were set up on the Swisshop server in Geneva to find additional copies of the same sequence, as they were deposited, and also copies of similar sequences. This became especially useful in 1997, since there were a large number of murine sequences deposited over the last year. (Swisshop is at http:\\www.expasy.ch\Swisshop Req.html.) Once an open reading frame had been identified, it was always necessary to obtain a cDNA clone before proceeding further, because of the large number of frameshifts and mis-sequences in the EST collection. Once the sequence had been confirmed, two approaches were used to identify the correct amino terminus. The first was to use artificial intelligence approaches, on the SignalP server (http:\\genome.cbs.dtu.dk\htbin\wwwsignalp). This was usually quite accurate, and gave either the correct amino terminus, or at least one which only differed by a couple of amino acids. However, our experience with RANTES ***(5)*** showed that in some cases getting the correct amino terminal sequence was very important if the biological function is to be correctly ascertained. Also, there are some cases, such as MIP-3 or MIP-5/HCC2 where there is a very long predicted signal sequence and the calculated signal peptidase cleavage site is not statistically significant ***(6)***. More recently we have expressed the cDNA in a transient expression system to experimentally determine the cleavage site for mammalian signal proteases ***(7)***. The mature proteins then can either be made by total chemical synthesis, or by expression in *E. coli*. Once protein has been obtained, it has been relatively straightforward to assign it some biological function. For example in the case of MIP-3α, the

observation that the protein was able to activate calcium transients in cell lines transfected with the orphan DCCR2 (soon to be renamed CCR6), led to the identification of a unique receptor ***(8)***. Similarly, the observation that synthetic MIP-3α could induce chemotaxis of CD34 derived dendritic cells and not monocyte derived dendritic cells led to hypotheses about the function of this receptor/ligand pair.

A current version of the chemokine alignment is shown in **Fig. 2**. Full listings of the known chemokines can be found at the Kumamoto University site (http: \\cytokine.medic.kumamoto-u.ac.jp\CFC\CK\chemokine.html). An earlier version of the figure is available at the Chemokine Information Source at http:\\www.expasy.ch\cgi-bin\chemokineTop.pl where there is an automated search within Swissprot for new names deposited for the existing sequences.

2.2. Pairing Up with the Orphans—and Explosion in Confirmed Chemokine Receptors

One of the most interesting events of 1997 in the chemokine world was the speed at which new chemokine receptors were classified over a period of a few months. CCR6 was identified as the MIP-3a/LARC receptor ***(8,9)***, CCR7 as the MIP-3β receptor ***(10)***, CCR8 as the I-309 receptor ***(9)*** and the CX_3CR1 as the receptor for Fractalkine ***(10)***. These receptors seem to have a higher degree of selectivity than the others previously identified. One initial response to this apparent selectivity is that it could simply be that the other ligands for these receptors are waiting to be identified. However, in all cases, at least 25 ligands have been tested, with only one being found to be positive. This compares with the CC chemokine receptors CCR1 through 5 where at least three ligands are found for each when a similar set of ligands are tested. The new chemokine receptor ligand pairs are interesting, since many of the ligands are located away from the main chemokine gene cluster on chromosome 17q11.2 and the receptors are located away from the receptor cluster on chromosome 3. Taken together, these results have been interpreted as implying a much more specific role for the recruitment and activation of particular sets of lymphocytes in specialized micro environments ***(4)***.

2.3. New Chemokines in the Viral World—Herpesviruses

It has been known for some time that herpesviruses encode functional chemokine receptors ***(11)***. One of the theories for the existence of such receptors is that they play an important part in allowing the virus to disseminate itself in vivo (*see* **ref.** ***12*** for a review). In late 1996, this picture was complicated further when automated searches for novel chemokines, such as those described above, uncovered two or three new chemokines in herpesvirus 8,

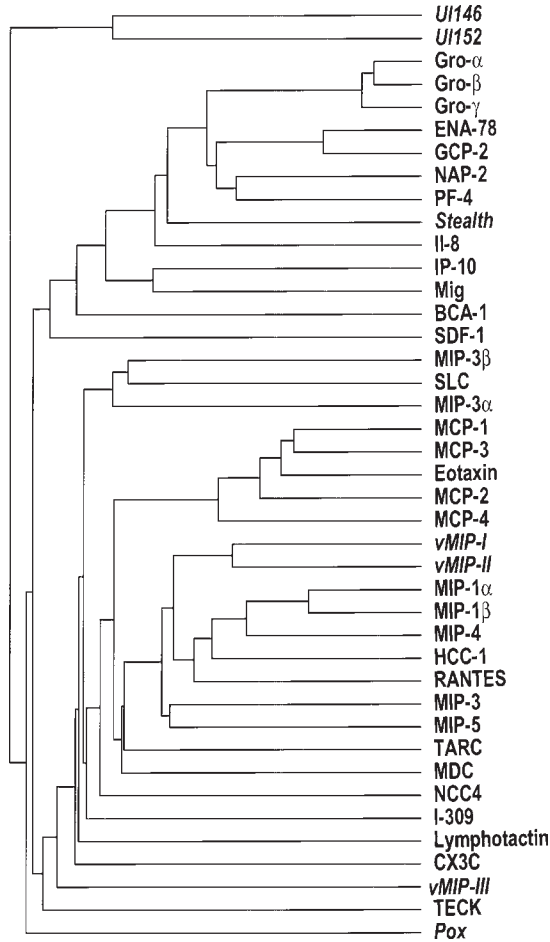

Fig. 2. The Chemokine Family. Dendogram showing the similarities between the human and virally encoded chemokine protein sequences. The sequences cluster in terms of the level of identity pairs of amino acids. The further to the right that the branch points are shown on the diagram, the more similar the two proteins are.

which is also known as Kaposi's sarcoma herpesvirus. The biological properties of these proteins are the basis of much study at this stage *(7)*, but there is a consensus that they are modulating the host immune response, and promoting neovascularization, to allow growth of the sarcoma itself. Interestingly, the searches also showed up an EST from a clinical isolate of a cytomegalovirus (CMV) strain isolated from a patient with chronic fatigue syndrome. This has a high degree of sequence identity with the human Gro-α gene (***13***; *see* **ref.** ***12*** for a discussion of the gene). Although the function of this gene is not clear at

this stage, it brought to light work on clinical isolates of CMV which showed that clinical strains of CMV contain many genes not found in laboratory strains ***(14)***. In all our work in the area of chemokines and viruses (both the CMV and the HIV viruses)—this served to underline that the wild-type, clinical isolates, and the laboratory adapted strains differ in the way they use the chemokine system. This may explain differences in virulence and tissue tropism, in vivo effects which are not selected for in laboratory culture systems.

3. Conclusions

One of the interesting questions is why this approach has not been reported to have been used to successfully identify new members of other families of cytokines, such as the four helix bundle family which includes IL-2, IL-4, IL-5, etc. One problem for these families is that the defining features are not so apparent (for example the positions of the disulfide bonds are not always conserved). Also, the majority of the members of these cytokine families are only finally confirmed once their three-dimensional structures have been solved. It may be that when more sophisticated versions of such techniques as Profile searching can be used will this then open up new cytokines for more classical families. Such Profiles would have to include amino acid similarities, as well as secondary structure propensity. Even so, the current rate of success is not expected to be as high as for the chemokine area (*see*, for example, **ref. *15***).

The availability of large amounts of raw sequence data, due to public EST collections, and the ability of scientists to access this data, has led to a massive increase in the number of novel proteins identified *in silico*—that is purely on computational techniques. Nowhere has this been as fruitful as in the chemokine area where there has been an explosive increase in the number of chemokines identified. The challenge now is to piece together the roles that these new chemokines play in routine immunosurveillance and also in inflammatory diseases. This challenge has been taken up in the last year with the identification of ligands for four chemokine receptors previously classified as orphans. In terms of receptor function, we still have a long way to go, since there are still many chemokines for which receptors have not been identified, but at least we have a more complete list of the players as far as the ligands are concerned.

On another level, the speed at which the data has become available for novel chemokines gives us a hint of how the future of biology will develop in the Web age. In one sense the revolution in bioinformatics means that more and more data is available to the scientific community, before it appears in publications based on paper technology. In another sense, the chemokine area has shown that it is relatively easy to obtain sequence information—by far the largest challenge in biology remains trying to work out the function of the proteins encoded by these sequences, and working out which ones are really important.

Acknowledgments

We thank all our colleagues in Geneva and elsewhere who have worked with us to help us understand the biological function of chemokines, but especially Christine Power and Amanda Proudfoot.

References

1. Tashiro, K., Tada, H., Heiker, R., Shirozu, M., Nakano, T., and Honjo, T. (1993) Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. *Science,* **261,** 600–603.
2. Imai, T., Baba, M., Nishimura, M., Kakizaki, M., Takagi, S., and Yoshie, O. (1997) The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. *J. Biol. Chem.* **272,** 15,036–15,042.
3. Wells, T. N. C. and Peitsch, M. C. (1997) The chemokine information source: identification and chracterization of novel chemokines using the WorldWideWeb and expressed sequence tag databases. *J. Leuk. Biol.* **61,** 545–550.
4. Yoshie, O., Imai, T., and Nomiyama, H. (1997) Novel lymphocyte-specific CC chemokines and their receptors. *J. Leuk. Biol.* **62,** 634–644.
5. Proudfoot, A. E., Power, C. A., Hoogewerf, A. J., Montjovent, M. O., Borlat, F., Offord, R. E., and Wells, T. N. C. (1996) Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist. *J. Biol. Chem.* **271,** 2599–2603.
6. Coulin, F., Power C. A., Alouani, S., Peitsch M. C., Schroeder J.-M., Moshizuki, M., Clark-Lewis, I., and Wells, T. N. C. (1997) Characterisation of macrophage inflammatory protein-5/human CC cytokine-2, a member of the macrophage-inflammatory-protein family of chemokines. *Eur. J. Biochem.* **248,** 507–515.
7. Kledal, T. N., Rosenkilde, M. M., Coulin, F., Simmons, G., Johnsen, A. H., Alouani, S., Power, C. A., Luttichau, H. R., Gerstoft, J., Clapham, P. R., Clark-Lewis, I., Wells, T. N. C., and Schwartz, T. W. (1997) A broad-spectrum chemokine antagonist encoded by Kaposi's sarcoma-associated herpesvirus. *Science* **277,** 1656–1659.
8. Power, C. A., Church, D. J., Meyer, A., Alouani, S., Proudfoot, A. E. I., Clark-Lewis, I., Sozzani, S., Mantovani, A., and Wells, T. N. C. (1997) Cloning and characterization of a specific receptor for the novel CC chemokine MIP-3α from lung dendritic cells. *J. Exp. Med.* **186,** 825–835.
9. Greaves, D. R., Wang, W., Dairaghi, D. J., Dieu, M. C., de Saint-Vis, B., Franz, K., Rossi, D., Caux, C., McClanahan, T., Gordon, S., Zlotnik, A., and Schall, T. J. (1997) CCR6, a CC chemokine receptor that interacts with macrophage inflammatory protein 3α and is highly expressed in human dendritic cells. *J. Exp. Med.* **186,** 837–844.
10. Yoshida, R., Imai, T, Hieshima, K., Kusssuda, J., Baba, M., Kitura, M., Nishimura, M., Kakizaki, M., Nomiyama, H., and Yoshie, O. (1997) Molecular cloning of a novel human CC chemokine EBI1-ligand chemokine that is a specific functional ligand for EBI1, CCR7. *J. Biol. Chem.* **272,** 13,803–13,809.

11. Gao, J.-L. and Murphy, P. M. (1994) Human cytomegalovirus open reading frame US28 encodes a functional β chemokine receptor. *J. Biol. Chem.* **269,** 28,539–28,542.
12. Wells, T. N. C. and Schwartz, T. W. (1997) Plagiarism of the host immune system: lessons about chemokine immunology from viruses. *Curr. Opinion Biotech.* **8,** 741–748.
13. Martin, W. J., Zeng, L. C., Ahmed, K., and Roy, M. (1994) Cytomegalovirus-related sequence in an atypical cytopathic virus repeatedly isolated from a patient with chronic fatigue syndrome. *Am. J. Pathol.* **145,** 440–451.
14. Cha, T.-A., Tom, E., Kemble, G. W., Duke, G. M., Mocarski, E. S., and Spaete, R. R. (1996) Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. *J. Virol.* **70,** 78–83.
15. Wells, T. N. C., Graber, P., Proudfoot, A. E. I., Arod, C. Y., Jordan S. R., Lambert, M. H., Hassel, A. M., and Milburn, M. V. (1994) The three-dimensional structure of human interleukin-5 at 2.4 angstroms resolution: implication for the structures of other cytokines. *Ann. NYAS* **725,** 118–127.
16. Peitsch, M. C. (1995) Protein modeling by E-mail. From amino acid sequence to protein structure: a free one-hour service. *Bio/Technol.* **13,** 658–660.
17. Peitsch, M. C. (1996) ProMod and Swiss-model: internet-based tools for automated comparative protein modelling. *Biochem. Soc. Trans.* **24,** 274–279.

8

Purification of Recombinant Chemokines from *E. coli*

Amanda E. I. Proudfoot and Frédéric Borlat

1. Introduction

The majority of chemokines are highly basic, small proteins, with a molecular mass of around 8–10 kDa. Although they do not necessarily have a high level of homology at the primary sequence level, which can be as low as 20%, although it can also be as high as 90%, the three-dimensional structure of all the chemokines solved to date has shown that they have a superimposable monomeric fold. This fold is imposed on the family by the four cysteine motif that is common to the majority of the chemokines since they all form two disulfides between Cys-1 and 3, and Cys2 and 4, whether they belong to the α or CXC or β or CC subclass. Molecular modeling of the two chemokines that deviate from the motif, the C chemokine lymphotactin which lacks a disulfide, and the CX_3C chemokine neurotactin or fraktalkine which has three amino acids between the first cysteine pair, both adopt the same fold. Modeling in fact shows that either one or three residues between the first two Cys can be adopted, but not two, probably explaining why examples of CX_2C chemokines do not exist.

Chemokines share another property which has been of help in their purification especially from natural sources (*see* Chapter 1 and ***[1–4]*** in that they all bind strongly to glycosoaminoglycans [GAGs]). Thus, they will bind to Heparin columns, and this property has been exploited in the purification of recombinant chemokines as well as the natural proteins. Since the chemokines are mostly highly basic with pI values around 9, this interaction with GAGs could be attributed to electrostatic interactions. However, MIP-1α, which has an acidic pI of 4.7, yet still binds to Heparin columns, albeit less strongly than

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others such as RANTES or MCP-1 which have pI values of 9.2 and 9.7, respectively. The principal region responsible for the GAG interaction has been shown to be the carboxy terminal portion, and is mediated by the basic Arg and Lys residues ***(5)***

This chapter will describe the purification and renaturation of recombinant proteins expressed heterologously in the procaryotic host *E. coli*. The most problematic step in producing chemokines recombinantly in the *E. coli* host is the expression itself, which is discussed in Chapter 4. In our hands, the chemokines were expressed uniquely as insoluble proteins in inclusion bodies, but they can be expressed as refolded soluble proteins in the periplasm (*see* Chapter 5). After cell breakage, and separation of the inclusion bodies from the soluble fraction by centrifugation at 10,000*g* for 60 min, SDS/PAGE analysis showed that there was in some cases recombinant protein in the soluble fraction. However, gel filtration of the soluble fraction in physiological buffers and analysis by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS/PAGE), showed that the protein was in fact aggregated, and required purification by the same method as the inclusion body protein. Expression in inclusion bodies allowed the isolation of the recombinant protein in a highly purified state after a single gel filtration step in a denaturing buffer of guanidine/HCl due the low molecular mass of the chemokines. Again probably due to their small size, the chemokines renatured easily and in high yield from the denaturant on dilution into Tris/HCl buffer. Renaturation can be monitored by a shift in the retention time on reverse phase high-performance liquid chromatography (HPLC), and by electrospray ionization mass spectroscopy since the oxidized protein has four mass units less than the reduced form due to the formation of the two disulfide bonds.

After renaturation, the majority of the recombinant chemokines are easily quantified by UV spectroscopy, although there are examples of chemokines such as NAP-2, which do not possess an aromatic amino acid that serve as chromophores. In this case, quantification is achieved by comparison of the peak height on reverse phase HPLC analysis to that of a known concentration of another chemokine. They can then be lyophilized after a change of buffer into a trifluoroacetic acid or acetic acid solution, which facilitates their storage as lyophilized powders. It is important that they are redissolved in water, before dilution into buffer or medium. Their handling is easy and rapid, as they are instantly soluble at concentrations as high as 1 m*M* if necessary, in aqueous solutions.

The methods described below have been applied to several chemokines including CTAP-III and NAP-2 ***(6)***, IL-8 ***(7)***, RANTES ***(8)***, MIP-1α ***(9)*** and MCP-1. When the cDNA encoding for the mature sequence of RANTES was

expressed the initiating methionine was retained, which resulted in a receptor antagonist, Met-RANTES ***(10)***. In order to obtain the correct amino terminal for the functional RANTES protein, a fusion construct was used, as suggested to us by PeproTech. The purification of the RANTES fusion was identical to that used for Met-RANTES. We have produced different cleavage sites to remove the leader sequence (*see* Chapter 4) since most of the chemokines that we have expressed with this leader sequence are susceptible to internal cleavage of arginine residues by Arg-C, with the exception of RANTES.

2. Materials

1. *E. coli* cells expressing the appropriate vector.
2. A fermentor or a shaker unit for shake flask cultures in a 37°C incubator.
3. Polytron homogenizer.
4. French pressure cell unit for cell breakage, maximum 500 mL of cell suspension used for small scale, or the Mantin Gaulin which can be used for volumes ranging from 0.5–4.5 l.
5. Centrifuge such as a Sorval (Dupont) to allow centrifugations at 10,000*g*.
6. Protein chromatographic system such as the FPLC (Pharmacia) system or Biologic System Controller (Bio-Rad, Richmond, CA), or equivalent equipment consisting of: Peristaltic pump; Fraction collector; UV detector; Chart recorder.
8. Magnetic heated stirrer.
9. Gel filtration column: 5 cm diam × 100 cm for large scale packed with a resin such as Sephacryl 200 HR or equivalent, prepacked Superdex 200 1.6 cm diam × 60 cm for small scale (Pharmacia) (*see* **Note 1**).
10. Cation exchange column of Hiload SP Sepharose HR, 26/10 (2.6 cm diam × 10 cm) or 16/10 (1.6 cm diam × 10 cm) (Pharmacia) (*see* **Note 1**).
11. Anion exchange column of Hiload Q Sepharose HR, 2.6 cm diam × 10 cm (Pharmacia) (*see* **Note 1**).
12. Heparin Sepharose column (1.6 cm diam. × 10 cm).
13. Reverse phase HPLC Gold system (Beckmann, Fullerton, CA) or equivalent equipped with a C8 Aquapore RP-300 7μm (0.2 cm diam. × 22 cm) (Applied Biosystem) for analytical applications or VarioPrep Nucleosil-300 5 μm C8 (1.0 cm diam. × 25 cm) (Macherey-Nagel) for preparative applications.
16. SDS/PAGE gel electrophoresis system (Bio-Rad, Novex, San Diego, CA or equivalent) supplied with gradient gels of 4–20% or homogeneous gels of 20% (*see* **Note 2**).
17. pH meter.
18. Conductivity meter.
19. Freeze-dryer.
20. Endoproteinase Arg-C (Boerhinger Mannheim).
21. Laboratory reagents from Sigma or Fluka, Buchs, Switzerland.

3. Methods

3.1. Buffers

1. Cell breakage buffer: 50 m*M* Tris-HCl, pH 8.0, 10 m*M* $MgCl_2$, 5 m*M* benzamidine/HCl, 1 m*M* dithiotreitol (DTT), 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF), 20 mg/L DNase (Fluka).
2. Inclusion body extraction buffer: 6 *M* Guanidine/HCl (*see* **Note 3**); 0.1 *M* Tris/HCl, pH 8.0; 1 m*M* DTT.
3. Gel filtration buffer: 6 *M* Guanidine/HCl; 0.1 *M* Tris/HCl, pH 8.0; 1 m*M* DTT.
4. Renaturation buffer (see **Subheading 3.3.**): 0.1 *M* Tris/HCl pH 8.0; 0.01 m*M* Oxidixed Glutathione; 0.1 m*M* Reduced Glutathione.
5. Cation exchange chromatography buffers: Buffer A: 50 m*M* sodium acetate, pH 4.5. Buffer B: Buffer A containing 2 *M* NaCl.
6. Anion exchange chromatography buffers: Buffer A: 50 m*M* Tris-HCl, pH 8.0. Buffer B: Buffer A containing 2 *M* NaCl.
7. Heparin sepharose chromatography buffers: Buffer A: 25 m*M* Tris-HCl, pH 8.0; 50 m*M* NaCl. Buffer B: Buffer A containing 2 *M* NaCl.
8. Hydrophobic interaction chromatography buffer: Buffer A: 50 m*M* sodium phosphate, pH 7.0, containing 1.7 *M* ammonium sulfate (*see* **Note 4**). Buffer B: 50 m*M* sodium phosphate, pH 7.0.
9. Reverse phase HPLC buffers: Buffer A: 0.1% trifluoroacetic acid. Buffer B: Buffer A containing 90% acetonitrile.
10. Arg-C buffer: 50 m*M* Tris-HCl, pH 8.0, 10 m*M* $CaCl_2$, 5 m*M* EDTA, 50 m*M* DTT.

3.2. Cell Breakage and Inclusion Body Extraction

The *E. coli* cell paste is suspended in a volume corresponding to three times the weight of the cell paste, e.g., 50 g *E. coli* cell paste (wet weight) is suspended in 150 mL breakage buffer. The cells are broken by three passages though a French pressure cell, with 30 s sonication on ice after each passage. (*see* **Note 5**). The resulting suspension is centrifuged for 60 min at 10,000*g*. The pellet (inclusion bodies) is solubilized in the inclusion body extraction buffer first by homogenization with a Polytron homgenizer, followed by stirring with the aid of a magnetic stirrer heated at 60°C for 30 min. This heating step is essential, even if the inclusion bodies are soluble in the guanidine buffer at room temperature to ensure monomerization of the aggregates that can form in inclusion bodies. The solution is allowed to cool to room temperature and is then applied to a gel-filtration column 100 mL aliquots on the 5 cm diam × 100 cm column of Sephacryl 200 HR equilibrated in the gel filtration buffer. The column is run at a flow rate of 2 mL/min and 20 mL fractions are collected with an elution volume of 2 L (**Fig. 1**).

The solubilized inclusion bodies obtained from small scale purifications yielding approx 20–30 *g E. coli* cell pastes (12 × 800 mL shake flask cultures)

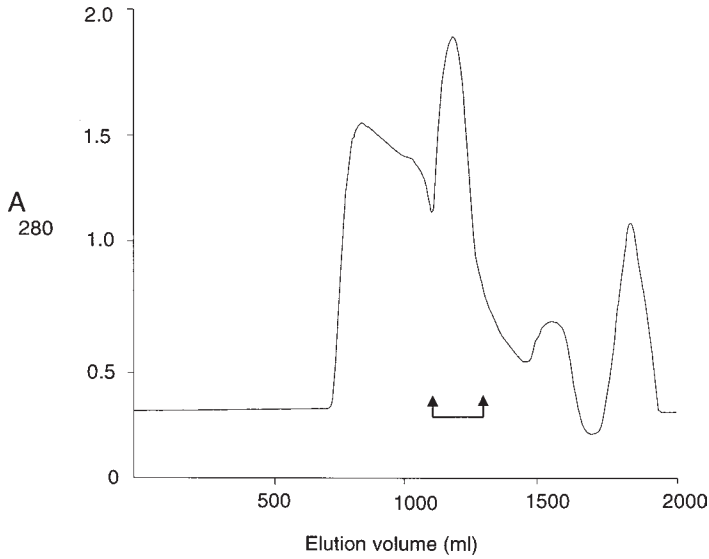

Fig. 1. Gel-filtration of the solubilized inclusion bodies from *E. coli* cells expressing Met-RANTES. 100 mL were loaded onto a Sephacryl 200 HR column (5 cm diam × 100 cm) and the elution carried out at a flow rate of 2 mL/min as described in **Subheading 3.2.** The fractions were pooled as indicated.

are applied in 2 mL volumes to a Superdex 200 HR column, equilibrated in the gel filtration buffer. 10 mL fractions are collected, and the flow rate is set at 1 mL/min with an elution volume for 120 mL, using the fast protein liquid chromatography (FPLC) system (*see* **Note 6**).

The chemokine containing fractions are identified by SDS/PAGE electrophoresis. Since the highly ionic nature of the guanidine/HCl buffer does not allow application of the samples directly, the samples are dialysed for 1 h against a solution of 6 *M* urea prior to the addition of an equal volume of sample buffer. Alternatively the proteins may be precipitated by the addition of an equal volume of ethanol (stored at –20°C) in Eppendorf tubes and allowed to stand at –20°C for 1 h. The tubes are then centrifuged for 2 min using a bench centrifuge, the supernatant removed, and the pellets dissolved in sample buffer. Electrophoresis is carried out according to the manufacturer's instructions (**Fig. 2**) (*see* **Note 7**).

3.3. Protein Renaturation and Concentration

3.3.1. Optimization of Redox Conditions

We have tested several variations of redox maintaining additives to optimize the renaturation yield. These include 1,4-dithio-DL-threitol (DTT) to maintain

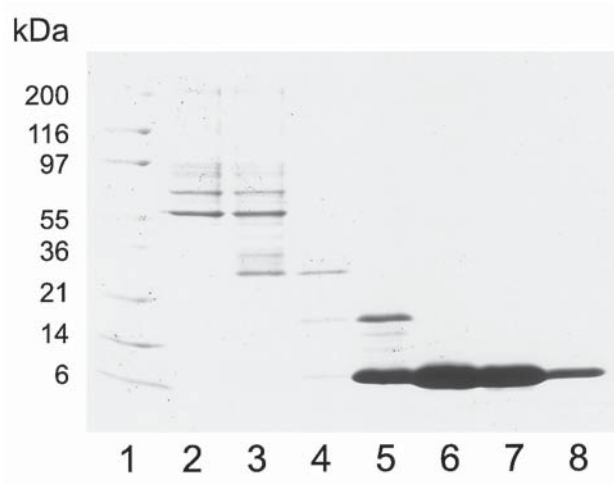

Fig. 2. SDS/PAGE analysis of the Sephacryl 200 HR chromatography. The samples were dialysed for 1 h against 6 *M* urea prior to SDS/PAGE analysis. Lane 1, molecular weight standards with masses as indicated; Lanes 2–10, fractions taken at 800, 900, 1000, 1100, 1160, 1240, and 1400 mL, respectively.

a reducing environment and varying ratios of oxidized and reduced glutathione to produce rapidly oxidizing as well as those that allow oxidation to occur more slowly. The results are summarized in **Table 1** for the renaturation of Met-RANTES, and we have routinely adopted the optimal conditions obtained described in **Subheading 3.1.** for all of our chemokine purifications. We do not however exclude that other conditions may be preferable for certain chemokines.

The proteins are renatured by a 10-fold dilution into renaturation buffer. The guanidine/HCl solution is added drop-wise into the renaturation buffer. If the volume is large, this can be carried out using a peristaltic pump. The solution is stirred overnight at 4°C. The solution often appears cloudy due to the precipitation of protein that has not renatured, and the solution is therefore centrifuged at 10,000*g* for 30 min. The supernatant containing the renatured protein is adjusted to pH 4.5 with acetic acid and the conductivity lowered to 20 mS or less by the addition of H_2O. The solution is then applied to a cation exchange resin previously equilibrated in 50 m*M* sodium acetate buffer, pH 4.5. The absorbed protein is eluted with a linear 0–2 *M* NaCl gradient in the same buffer (**Fig. 3**). In the case of MIP-1α which has a pI of 4.7, concentration is carried out at pH 8.0 on an anion exchange resin equilibrated in 20 m*M* Tris/HCl, pH 8.0, and elution carried out by a linear 0–0.5 *M* NaCl gradient in anion exchange buffer.

Table 1
Yields Obtained for Different Renaturing Conditions for Met-RANTES[a]

Redox reagent	Renaturation yield (%)
None	15
1 m*M* GSSG and 0.1 m*M* GSH	45
0.1 m*M* GSSG and 0.01 m*M* GSH	35
0.1 m*M* GSSG and 1 m*M* GSH	55
0.01 m*M* GSSG and 0.1 m*M* GSH	61
1 m*M* DTT	16
5 m*M* DTT	25

[a]The renaturations were carried out in 20 m*M* Tris/HCl with the following redox reagents added: GSSG, oxidized glutathione; GSH, reduced glutathione; DTT,1,4-Dithio-DL-threitol.

If several purifications are carried out simultaneously as in the case of alanine scanning mutagenesis studies, the solutions containing the renatured mutants can be applied to small columns packed with the ion exchange resin. We found that plastic columns (2.5 cm diam × 10 cm) supplied by Bio-Rad ideal for this purpose, which are filled to a height of approx 5 cm. The solutions are applied by gravity, and after washing with 2–5 column volumes of the equilibration buffer, elution can be carried out by a single step with buffer containing 2 *M* NaCl of if preferred with 3 steps of 0.5 *M*, 1 *M* and 2 *M* NaCl.

The fractions containing the protein are identified by SDS/PAGE electrophoresis and stained by Coomassie Blue. The fractions are pooled, and dialyzed against the appropriate buffer if an additional purification step of either hydrophobic interaction chromatography or Heparin Sepharose chromatography (*see* **Subheading 3.4.1.** and **3.4.2.**) is required. If the protein is sufficiently pure, or if reverse phase HPLC is required (*see* **Subheading 3.4.3.**), the protein is dialyzed against two changes of 1% acetic acid, and one change of 0.1% trifluoroacetic acid (TFA). After dialysis, the solution is shell frozen in a freezing bath of ethanol containing dry ice, and lyophilized.

3.4. Additional Purification Steps

Three different chromatography steps can be used for removing any protein contaminants.

3.4.1. Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography is carried out by applying the solution to Phenyl Sepharose columns (26/10 or 16/10) equilibrated in 50 m*M*

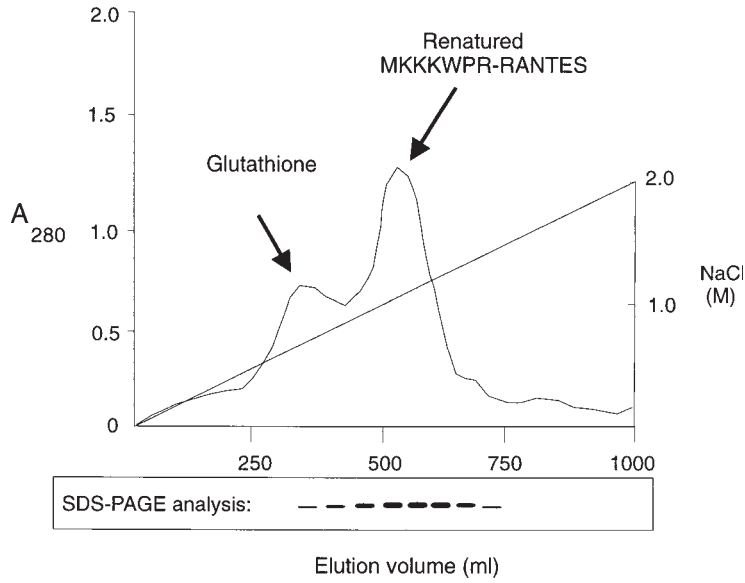

Fig. 3. Elution profile of the renatured RANTES fusion protein on a Hiload SP Sepharose HR 26/10 column. The protein was eluted with a linear gradient of 0–2 *M* NaCl as described in **Subheading 3.3.1.** and 10 mL fractions collected. The result of SDS/PAGE analysis is shown in the insert, showing that the first peak is due to the UV absorbance of glutathione.

sodium phoshate containing 1.7 *M* ammonium sulfate. The solution must be adjusted to pH 7.0, and ammonium sulfate added to 1.7 *M* prior to loading (*see* **Note 4**). The column is washed with two column volumes of the equilibration buffer, and the adsorbed proteins eluted with a linear 1.7–0 *M* gradient of ammonium sulfate. The ammonium sulfate does not interfere with SDS/PAGE analysis. The pooled fractions are subsequently dialyzed against two changes of 1% acetic acid, one change of 0.1% TFA and lyophilized.

3.4.2. Heparin Sepharose Chromatography

The protein solution, previously dialyzed against 25 m*M* Tris/HCl, pH 8.0 containing 50 m*M* NaCl, or adjusted to pH 7.0 and NaCl added to 50 m*M*, is applied to the Heparin Sepharose column. The coulmn is washed with two volumes of loading buffer, and the adsorbed protein eluted with a linear gradient of 0.05–1 *M* NaCl. The pooled fractions are dialyzed and lyophilized as described above.

3.4.3. Reverse Phase HPLC Chromatography

The lyohilized protein is dissolved in 0.1% TFA. The C8 Aquapore RP-300 7 μm (0.2 cm diam. × 22 cm) column may be used for quantities less than 0.5 mg, whereas 2 mg can be applied to the Nucleosil-300 5μm C8 (1.0 cm diam. × 25 cm) column. Absorbance is monitored at 214 n*M* and the fractions collected either manually or with a fraction collector.

3.5. Cleavage of Fusion Constructs

3.5.1. Arg-C Cleavage of the RANTES Fusion

The hexapeptide leader sequence was removed from the fusion protein by incubation in 50 m*M* Tris/HCl buffer, pH 8.0, with Endoproteinase Arg-C (1 : 100, enzyme:substrate, w/w), for 3 h at 37°C or overnight at 37°C (1 : 600, enzyme:substrate, w/w). Cleavage can be monitored by analytical reverse phase HPLC, where a small decrease in retention time is observed for the cleaved product (**Fig. 4**). The appearance of peptides with low retention times resulting from cleavage of the MKKKWPR sequence are observed. The cleaved product is separated by cation exchange chromatography as described in **Subheading 3.3.1.** except that 6 *M* urea was included in both buffers (**Fig. 5**). The fractions containing the cleaved RANTES protein are identified by SDS/PAGE analysis, pooled and dialyzed and lyophilized as previously described in **Subheading 3.3.1.**

3.5.2. Factor-Xa Cleavage

The fusion protein is incubated in 50 m*M* Tris-HCl, pH 8.0, with Factor Xa (1 : 50, enzyme : substrate, w : w) at 37°C overnight and purified as described in **Subheading 3.5.1.**

3.5.3. CNBr Cleavage

The protein is dissolved in 70% formic acid and a solution of CNBr in 70% formic acid added to achieve a 100-fold molar excess of CNBr. The solution is incubated overnight in the dark at room temperature in a large lyophilization flask. The solution is diluted 10-fold (*see* **Note 8**), shell frozen, and lyophilised. The cleaved product is purified as described in **Subheading 3.5.1.**

3.6. Preparation of Protein for In Vivo Studies

Because the chemokines have been produced in *E. coli*, they are not suitable for in vivo studies unless specially treated. A passage on a reverse-phase HPLC column will remove bacterial lipopolysaccharide (LPS). If large amounts are required, we have found that LPS can be removed by a hydrophobic interaction chromatography step on a Phenyl Sepharose column provided the follow-

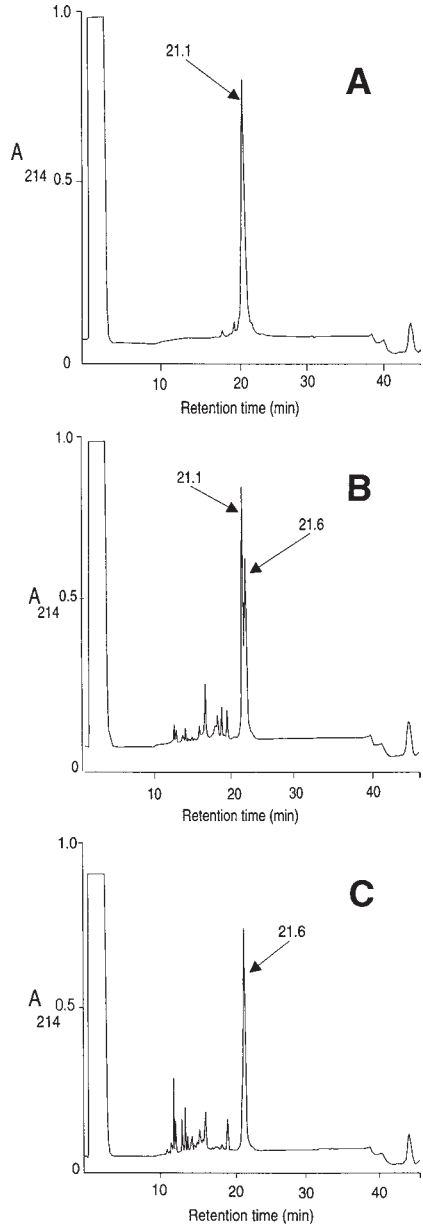

Fig. 4. HPLC analysis of the Arg-C cleavage of the RANTES fusion construct. The MKKKWPR-RANTES protein was digested for 3 h at 37°C using an enzyme:substrate ratio of 1 : 100 as described in the text. The fusion protein elutes with a retention time of 21.1 min and RANTES elutes at 21.6 min. **(A)** before addition of Arg-C; **(B)** 1 h digestion; **(C)** 3 h digestion.

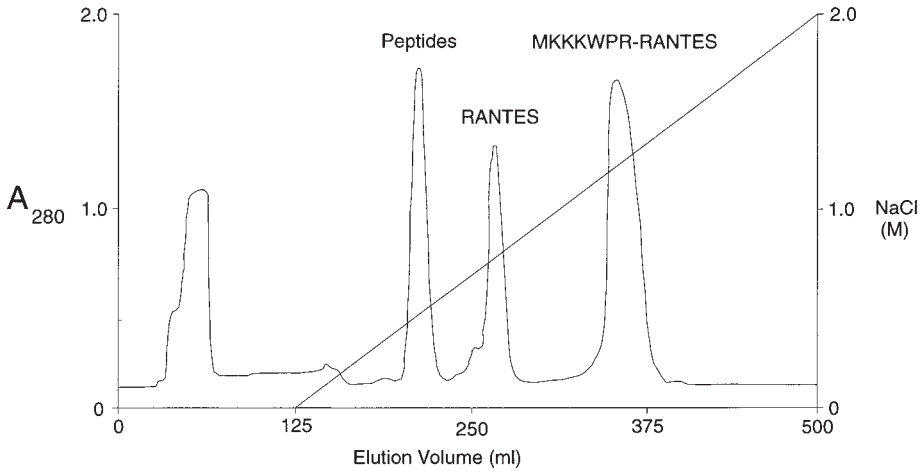

Fig. 5. Purification of an incomplete Arg-C digestion of the RANTES fusion. The solution was adjusted to 6 *M* urea and applied to a Hiload SP Sepharose HR, 26/10 as described in the text. 10 mL fractions were collected.

ing precautions are taken. All glassware used should be sterile, and all buffers filtered with sterile 0.22-μm filters. The resin must be washed with 2 column volumes of 1 *N* NaOH, then with distilled water prior to equilibration. After the protein solution has been applied to the column, at least 20 cloumn volumes should be applied to ensure that all the LPS has been washed out before elution of the protein.

3.7. Analysis, Quantification, and Storage

Analysis of chromatographic steps, and the purity of the proteins are carried out by SDS/PAGE and stained with Coomassie Brilliant Blue R250. We have routinely used the Novex system with precast 4–20% acrylamide mini gels, but any appropriate system may be used. Although the mini gels are slightly more time-consuming than the Phast system (Pharmacia), we prefer the resolution of the former system. Purity is always controlled by analytical reverse-phase HPLC.

The final product is controlled by electrospray ionization mass spectroscopy. If the mass obtained does not correspond to that predicted for the chemokine with the formation of two disulfide bonds, amino terminal sequencing and amino acid composition analysis is used to identify possible mutations.

The amounts of total protein in crude extracts are estimated using a colometric assay such as the Coomassie Plus Protein assay reagent (Pierce) or equivalent. The amount of purified recombinant chemokine protein is determined by UV spectroscopy at 280 nm. The extinction co-efficient is calculated from the amino acid composition according to the formula:

$$\frac{(150 \times \text{S-S}) + (5700 \times \text{W}) + (1320 \times \text{Y})}{\text{MW}}$$

This can be automatically computed from the sequence by software programs such as ProtParam in Swiss-Prot. Thus, the amounts can be directly calculated using for example, $A_{1cm}^{0.1\%} = 1.6$ for RANTES and 1.28 for MIP-1α.

The purified proteins are dialyzed extensively against two changes of 1% acetic acid and finally against 0.1% trifluoroacetic acid They can be stored as lyophilized powders at –20°C or –80°C and are stable at room temperature for a few days. It is very important to dissolve them in H_2O and not buffers or medium, since on lyophilization they form the trifluoroacetate salt, but once in aqueous solution at low pH can be adjusted to the required physiological pH. We routinely dissolve them in H_2O at a concentration 10-fold higher than required, and dilute 10-fold into the required buffer or medium. This does not pose a problem even for micromolar concentrations, since all of the chemokines that we have worked with are soluble at 1.25 m*M* or 10 mg/mL.

4. Notes

1. We have routinely used the prepacked chromatographic columns supplied by Pharmacia which are adapted for use for their FPLC system. However, the columns cited in this chapter are by no means the only ones that can be used. Columns of the appropriate size may be packed with equivalent resins which are available from several suppliers.
2. SDS/PAGE gel electrophoresis can be carried out on any appropriate system. Gels can be cast manually, but it is recommended to use high acrylamide concentrations for optimal separation of low molecular weight proteins. We have found that gradient gels give the optimal separation of proteins below 20 kDa.
3. Guanidine, if purchased as the hydrochloride salt, renders the aqueous solution acidic, and the guanidine is sparingly soluble. Therefore, the guanidinium/HCl is weighed out to attain the required molarity and the appropriate amount of solid Trizma base added. Dissolution is very rapid, and the pH is subsequently adjusted with HCl.
4. It is very important to adjust the protein solution to the same concentration of amonium sulfate as in Buffer A (1.7 *M*) before applying to the column to achieve adsorption of the proteins.
5. Cell breakage is controlled microscopically to ensure that it has been effective.
6. The elution volume, fraction size, and elution rate can be adapted for the system that is used.
7. We have recently found that for recombinant RANTES purifications the gel filtration step in guanidine/HCl can be replaced by dialysis against 1% acetic acid. The RANTES protein remains soluble while the contaminating proteins precipitate during the dialysis. The RANTES is recovered in the supernatant after cen-

trifugation, and following lyophilization, it is again dissolved in 6M guanidine buffer and renatured.

8. 70% formic acid solutions will not freeze so dilution is essential prior to lyophilization.

References

1. Schroder, J. M. (1997) Identification and structural characterization of chemokines in lesional skin material of patients with inflammatory skin disease. *Methods Enzymol.* **288,** 266–297.
2. Schroder, J. M. (1997) Isolation and purification of neutrophil-activating peptide-4: a chemokine missing two cysteines. *Methods Enzymol.* **287,** 216.
3. Noso, N., Proost, P., van Damme, J., and Schroder, J. M. (1994) Human monocyte chemotactic proteins-2 and 3 (MCP-2 and MCP-3) attract human eosinophils and desensitize the chemotactic responses towards RANTES. *Biochem. Biophys. Res. Commun.* **200,** 1470–1476.
4. Proost, P., Struyf, S., Couvreur, M., Lenaerts, J. P., Conings, R., Menten, P., Verhaert, P., Wuyts, A., and van Damme, J. (1998) Posttranslational modifications affect the activity of the human monocyte chemotactic proteins MCP-1 and MCP-2: identification of MCP-2(6-76) as a natural chemokine inhibitor. *J. Immunol.* **160,** 4034–4041.
5. Kuschert, G. S., Hoogewerf, A. J., Proudfoot, A. E. I., Chung, C. W., Cooke, R., Hubbard, R. E., Wells, T. N. C., and Sanderson, P. N. (1998) Identification of a glycosoaminoglaycan binding site on human interleukin-8. *Biochemistry* **37,** 11,193–11,201.
6. Proudfoot, A.E., Peitsch, M. C., Power, C. A., Allet, B., Mermod, J. J., Bacon, K., and Wells, T. N. (1997) Structure and bioactivity of recombinant human CTAP-III and NAP-2. *J. Protein Chem.* **16,** 37–39.
7. Alouani, S., Gaertner, H. F., Mermod, J. J., Power, C. A., Bacon, K. B., Wells, T. N., and A.E. Proudfoot, A. E. (1995) A fluorescent interleukin-8 receptor probe produced by targetted labelling at the amino terminus. *Eur. J. Biochem.* **227,** 328–334.
8. Proudfoot, A. E., Power, C. A., Hoogewerf, A., Montjovent, M. O., Borlat, F., and Wells, T. N. (1995) Characterisation of the RANTES/MIP-1 alpha receptor (CC CKR-1) stably transfected in HEK 293 cells and the recombinant ligands. *FEBS Lett.* **376,** 19–23.
9. Solari, R., Offord, R. E., Remy, S., Aubry, J. P., Wells, T. N., Whitehorn, E., Oung, T., and Proudfoot, A. E. (1997) Receptor-mediated endocytosis of CC-chemokines. *J. Biol. Chem.* **272,** 9617–9620.
10. Proudfoot, A. E., Power, C. A., Hoogewerf, A. J., Montjovent, M. O., Borlat, F., Offord, R. E., and Wells, T. N. (1996) Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist. *J. Biol. Chem.* **271,** 2599–2603.

9

Chemokine Receptor Cloning

Philip M. Murphy

1. Introduction

Detailed understanding of chemokine action ultimately requires molecular cloning of chemokine receptor genes and cDNAs. Both forward and reverse genetic methods have been used for this purpose ***(1)***. Forward methods, which involve primary purification of a protein based on a defined physical or functional property, has been used for only one chemokine receptor, the Duffy antigen receptor for chemokines, and was actually used before Duffy was shown to bind chemokines ***(2)***. Reverse methods, which involve cloning a cDNA or gene without primary knowledge of the structure of the encoded protein, can be divided into two types: expression and homology hybridization cloning.

Expression cloning can also be classified into two major types, based on RNA distribution or structure or function of the encoded protein. The latter involves iterative selection of a cDNA from a library of cDNAs based on acquisition of a physical or functional property specific to the encoded protein by a naive cell type recipient of the library or portions of it. Many assays can be used to detect the protein, including antibody binding, ligand binding, and a variety of signaling assays such as agonist-induced calcium flux and transmembrane current. With respect to leukocyte chemoattractant receptors, this type of expression cloning was used in pioneering studies in the early 1990s for cloning of cDNAs encoding the fMLP, C5a and PAF receptors and for the chemokine receptor CXCR1 ***(3–6)***. Later, the cDNA for CXCR4 was also identified by expression cloning, based on its HIV-1 coreceptor activity ***(7)***. Expression cloning by RNA distribution involves identification of a cDNA based on its expression pattern in tissues and cells, and typically relies on the technique of subtractive hybridization. CXCR5 and CCR7 were identified using this approach ***(8,9)***.

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All other chemokine receptors have been identified by homology hybridization. This technique requires knowledge of at least one other gene sequence, and exploits the fact that genes with similar function often have conserved sequences that may cross-hybridize. In the case of some protein families, chemokine receptors included, this technique has largely replaced the classic direct approach to gene characterization and greatly accelerated gene discovery. There are two general approaches to homology hybridization cloning, low-stringency blot hybridization, and polymerase chain reaction (PCR)-based cloning ***(10)***. The former approach was applied first to chemoattractant receptors by Gerard and Gerard (C5a receptor) and shortly thereafter by Navarro and coworkers and Murphy and Tiffany (CXCR1 and CXCR2, respectively) ***(11–14)***.The latter approach was first used by Neote et al. to clone CCR1 ***(15)***. This chapter will focus on both of these methods, since they have been the most widely used for chemokine receptor cloning.

One reason homology hybridization cloning has worked so well for chemokine receptors is that their primary sequences are conserved not only with each other but also with other types of G protein-coupled receptors ***(16)***. As a result, there have been both "willful" discoveries of novel chemokine receptors, by scientists investigating biological processes in which chemokines have been implicated mechanistically, such as immunity and inflammation, as well as "accidental" discoveries, mainly by neuroscientists and endocrinologists looking for G protein-coupled receptors specific for neurotransmitters or hormones. Sequence analysis programs quickly turn such accidents into opportunities for immunologists by quantitating the relatedness of a newly discovered sequence to those of known chemokine receptors. Rapid progress in chemokine discovery, now fueled by bioinformatics, mainly through analysis of expressed sequence tags (EST) ***(17)***, has also been responsible for the success in chemokine receptor discovery. As additional receptor candidates have been identified by homology hybridization cloning, there has been no shortage of novel chemokines to screen for specific interactions.

Chemokines possess definitive sequence motifs and are small and highly expressed in many tissues. In contrast, chemokine receptors are comparatively large and are expressed at relatively low levels in a tissue/cell type-restricted manner. These differential features favor EST cloning as a way to find new chemokines and homology hybridization cloning, particularly starting from genomic DNA libraries, as a way to identify novel chemokine receptors.

Sequence divergence among members of the G protein-coupled receptor superfamily is heterogenously distributed throughout the protein chain, with regions of highest similarity among distantly related proteins occurring in the transmembrane regions. Even highly divergent genes have been discovered by taking advantage of this property through the appropriate design of PCR

primers from highly conserved regions. As described in **ref. *1***, low stringency blot hybridization is useful mainly for cloning genes within a gene cluster (cluster walking) whereas PCR strategies can go beyond this to explore genes in other clusters (cluster jumping).

2. Materials

All buffers should be prepared from analytical grade reagents and dissolved in distilled water. The solutions can be stored at room temperature.

2.1. Low-Stringency Blot Hybridization

1. Full-length open reading frame DNA fragment from a known chemokine receptor.
2. cDNA or genomic DNA library with titer $>10^6$ plaque forming units (pfu)/μL. The methods here are based on a lambda bacteriophage library.
3. *E. coli* host strain (e.g., Y1088, Y1090, LE392).
4. Whatman 3MM paper, nylon transfer paper (e.g., Nytran, Schleicher and Schuell, Keene, NH).
5. 20% maltose; 1 *M* $MgSO_4$; agarose (not agar); Lennox L broth (LB; Quality Biological #340-016-100); SM media (150 m*M* NaCl, 10 m*M* Mg_2SO_4).
6. Random primer DNA labeling kit (Boehringer-Mannheim, Indianapolis, IN).
7. SSPE solution: 150 m*M* NaCl, 10 m*M* NaH_2PO_4, and 1 m*M* Na_2EDTA, pH 7.4.
8. Bacterial culture plates, 100 and 150 mm diameter.

2.2. PCR

1. Thermocyler.
2. PCR kit.
3. Oligonucleotide primer pairs.
4. Template DNA (genomic DNA, DNA from a cDNA library, RNA).

3. Methods

All steps in this protocol are adapted from standard methods in molecular biology ***(18)***.

3.1. Low-Stringency Blot Hybridization

3.1.1. Plating of Phage Library

1. Prepare top agarose (3.75 g agarose in 500 mL LB) and bottom agarose (7.5 g agarose in 500 mL LB). Autoclave each for 20 min, then maintain at 50°C until use. Pour 30 mL bottom agarose into 10–20 150 mm dishes. Dry plates upright with lids lifted in an unhumidified 37° or 42°C incubator for ~20 min just before use.
2. Grow bacteria to turbidity in 25–50 mL LB containing 10 m*M* Mg_2SO_4 and 0.2% maltose (~6 h). Pellet bacteria and resuspend in LB to a density given by $OD_{600} = 0.5$.

3. Infect 600 μL of bacterial suspension with 50,000–100,000 pfu from library stock in a 15 mL round-bottom tube.
4. Incubate at 37°C for 20 min, then add 9 mL of top agarose and mix *quickly* by inverting the tube three times. Pour quickly onto bottom agarose on 150 mm plate. Remove bubbles. Repeat for 10 plates (500,000–1,000,000 pfu total). After agarose has solidified, invert plates and incubate at unhumidified 42°C overnight to allow plaques to form. If the library titer is not known at the start, it can be determined by performing **steps 1–4** using 100 mm plates, 300 μL of bacteria, 4.5 mL of top agarose in 5 mL round-bottom tubes, and serial dilutions of the library. Back-calculation gives the titer.

3.1.2. Transfer and Immobilization of Phage DNA to Nitrocellulose Filters

1. Chill plates at 4°C for 1 h to harden the agarose and prevent separation of top from bottom layer. Label duplicate sets of 150 mm nitrocellulose filters for each plate.
2. Apply each filter to plate (2 min for the first, 5 min for the second). This is done by puckering the filter, placing the rounded end on the middle of the plate and allowing the sides to be drawn to the plate by surface tension as they moisten.
3. Define the location of the filter on the plate by puncturing the filter/agarose in three asymmetric locations with a needle dipped in India ink.
4. Peel off filters using blunt-ended forceps and immerse individually in a shallow tray, e.g., a cafeteria tray that accommodates multiple filters, containing denaturing buffer (0.5 *M* NaOH, 1.5 M NaCl) for 90 s.
5. Transfer the filter into a new tray containing neutralizing buffer (0.5 *M* Tris [pH 7.4], 1.5 *M* NaCl) for 5 min.
6. Rinse filters individually in 2X SSPE.
7. Dry filter, DNA side up and individually, by placing on Whatman 3 MM paper for ~15 min.
8. Bake filters in a vacuum oven at 80–90°C for 45–60 min with vacuum on. Multiple filters can be stacked together at this step.

3.1.3. Probe Labeling

Explicit Instructions can be found on Package Insert in DNA Labeling Kits

3.1.4. Hybridization

1. Stack 10 filters in a plastic "seal-a-meal" bag. Wet with 6X SSPE and then drain.
2. Add 10 mL hybridization buffer, (6X SSPE, 50% formamide, 0.5% SDS, 10% dextran, and 50 μg/mL of sonicated salmon sperm or herring sperm DNA). Remove air and bubbles and seal by heating the open side. Incubate at 37°C for 1 h, open bag by cutting one corner, and drain.
3. Add fresh 10 mL hybridization buffer to bag. Denature desired amount of probe (~10^6 cpm/mL hybridization buffer) by incubating in NaOH 0.1 *M* at room

temperature for 5 min. Add denatured probe directly to bag. Remove air and bubbles and seal by heating the open side.

4. Place bag in container 1/3 filled with water and place container in a shaking water bath at 37°C for at least 12 h.
5. Rinse filters in salt/detergent solution twice at room temperature, then perform final wash at higher temperature. The salt concentration and temperature used for the final wash are critical and define the screening "stringency." For low-stringency screens, the following wash conditions are often used: washing solution composed of 5X SSPE and 0.1% SDS; temperature = 45–55°C; and wash time = 30–60 min.
6. After the final wash, rinse the filters in fresh wash buffer, then place them between two layers of plastic wrap, fix to a Whatman 3MM support and expose to X-ray film, typically for at least 24 h if an intensifying screen is used. Phosphorimagers, if available, can be used to shorten the development time.
7. Positive signals are defined as those that occur on the same position of both filters lifted from the same plate. This can be determined by superimposing the X-ray image of each filter aligned using the system described in **Subheading 3.1.2., step 3**. At this stage, these are called primary plaques, and need to be purified. Purify primary plaque from surrounding negative plaques by excising the gel portion containing it and placing it into 1 mL of 10 m*M* $MgSO_4$. This can most easily be done by localizing the area on the plate corresponding to the positive signal on the X-ray film and jabbing the agarose with the fat end of a Pasteur pipet. If the plate is confluently lysed by plaques this amount of gel in 1 mL of buffer will typically release enough phage to give a titer of 80,000 pfu/μL.
8. Repeat above procedures using 100 mm plates, 4.5 mL agarose, 5 mL round bottom tubes, 300 μL of bacteria, and primary phage stock. Plate ~1000 plaques and repeat hybridization steps. This can be done using only one filter per plate. Pick positive plaques, which are now called secondary plaques, and place in 1 mL of 10 m*M* MgSO4. This gives a titer of ~1000 pfu/μL. Repeat procedure if necessary to isolate a pure positive plaque uncontaminated by surrounding negative plaques.
9. Positive phage are then amplified by adding an excess (>105 pfu/plate) to *E. coli* (repeat **Subheading 3.1.1., steps 3** and **4**) so that plates are confluently lysed. To recover the virions, 15 mL of SM are added to each plate and the plates are rocked at room temperature for 2–3 h. The supernatant is recovered and cleared by shaking vigorously after addition of 200 μL chloroform. This will lyse any bacteria present and release additional phage. Bacterial DNA and RNA are then degraded by adding Rnase (400 mg/mL) and Dnase I (100 units) to the suspension at 37°C for 1 h.
10. Concentration of phage particles. An equal volume of polyethylene glycol (20% in 2 *M* NaCl in SM) is added to the phage suspension, mixed well and placed on ice for 1 h. Polyethylene glycol can be obtained in various size ranges; we use an average molecular weight of 3,450 Da (P-3640 from Sigma, St. Louis, MO). Phage particles are then precipated by centrifugation at 10,000*g* for 30 min, and resuspended in 0.5 mL SM in a microfuge tube. To uncoat the phage genome add

5 μL of 500 m*M* EDTA and 5 μL of 10% SDS, and incubate at 65°C for 20 min. To purify the phage genome from protein, extract with 1:1 phenol, then 1:1 chloroform. To concentrate the phage DNA, add 2 vol ethanol and 0.1 vol 3 *M* sodium acetate. A strand of DNA should become visible immediately, and can be recovered by centrifugation. The pellet is washed with 70% ethanol, vacuum-dried, and then dissolved in 20 μL distilled water. Approximately 10-20 μg phage DNA can be purified starting with 1 confluently lysed 150 mm plate.

11. DNA fragments of interest can be isolated from the phage DNA by appropriate restriction enzyme digestions and hybridizations, and subcloned into expression vectors for sequencing and functional analysis. The specific methods for these techniques are standard and can be found in molecular biology methods manuals.

3.2. PCR

Transmembrane domains 2, 3, 6 and 7, and the nine amino acids following TMD3, DRYLAIVHA (2nd intracellular loop) have been particularly useful for PCR cloning of novel chemokine receptor genes and cDNAs because they are the most highly conserved. As a result, probes designed from these regions can have significantly higher specificity than full-length ORF probes. Several oligonucleotides can be used separately or mixed together to make a complex transmembrane domain-selective probe for plaque hybridization screening of genomic and cDNA libraries, or else pairs of degenerate sense and antisense oligonucleotides from various transmembrane domains can be used in PCR-coupled cross-hybridization cloning strategies. Specificity depends on the oligonucleotides used and the cellular source of the DNA template that is amplified. The relative position of the oligonucleotides in a known G protein-coupled receptor can quite reliably predict the size of the PCR product for a new receptor. Often the product will contain multiple distinct DNA fragments, some known, some new. The known ones can serve as internal positive controls for the experiment, and can be detected by diagnostic restriction fragments, specific hybridizations and/or DNA sequencing of the PCR product.

Restriction enzyme sites can be incorporated into the 5'-end of the oligonucleotides to facilitate subcloning of the PCR product. If a novel relevant sequence is identified, it can be labeled and used to obtain DNA clones from a library that contains the full-length open reading frame. PCR cloning is highly empirical and depends on fortunate choices of template and oligos, as well as reaction conditions. In particular, changing the annealing temperature in the PCR cycle will affect the range of genes that can be identified.

3.2.1. Oligo Design

1. Align a group of known chemokine receptor amino acid sequences (**Fig. 1**).
2. Identify two regions of 10 uninterrupted amino acids having >75% identity and separated by at least 200 nucleotides (e. g., regions of transmembrane domains 2,

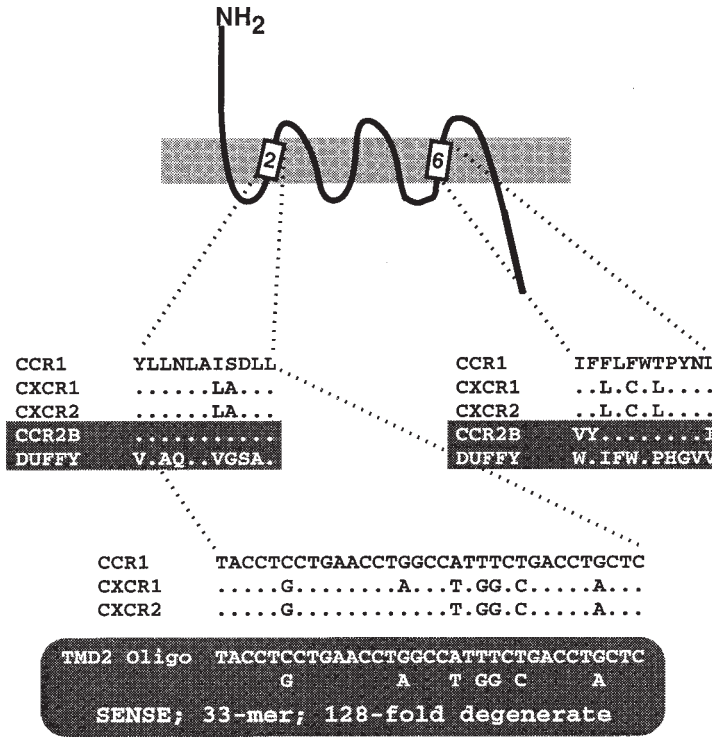

Fig. 1. Cloning by PCR. Highly conserved sequences of known chemokine receptors or other G protein-coupled receptors can be used to design biased, degenerate oligonucleotides that can be used for cross-hybridization to DNA libraries either by plaque hybridization or PCR methods. Examples for transmembrane domains (TMD) 2 and 6 are shown, first aligning the indicated amino acid sequences from both TMD2 and TMD6, and then aligning the corresponding DNA sequence for TMD2 (dots indicate position identities with the CCR1 sequence). Degenerate oligonucleotides were made based only on the CXCR1, CXCR2 and CCR1 sequences (only the TMD2 oligo is shown). They are able to cross-hybridize to CCR2 but not to Duffy sequences (note the highly conserved CCR2 and highly divergent Duffy sequences in the middle panel, black boxes). This example illustrates the possibilities and limitations for discovery of chemokine receptor genes by cross-hybidization (modified from **ref. *1***).

3, 6, and 7). The one that is most N-terminal will be used to design a 5'-oligo, the one that is most C-terminal will be used to design a 3'-oligo for PCR.

3. Align the corresponding DNA sequences.
4. Synthesize a specific oligonucleotide that contains all of the sequence possibilities of the aligned sequences. DNA synthesizers are capable of multiple nucleotide additions at each position, which "degenerates" the position. Oligos that are as much as 1048-fold degenerate have been used successfully in PCR cloning

strategies. When a position in the alignment calls for all four nucleotides, inosine can be used as a general surrogate.

3.2.2. PCR Cloning

1. Add 5' and 3' oligos (1 μ*M* each) with suitable template (50 ng cDNA or genomic DNA), in 100 μL total vol containing 200 μM of each nucleotide and 2 units of a thermostable DNA polymerase (e. g., Taq or Pfu DNA polymerase). Appropriate buffers are supplied by polymerase manufacturers. Annealing and elongation temperatures can be reduced to favor amplification of related sequences. As one of many examples of success, Neote et al. ***(15)*** succeeded in amplifying CCR1 and several related sequences using amplimers targeting TMD2 (5') and the conserved DRYLAIVHA motif that follows TMD3 (3'), and 2 μg of total monocyte and B-cell RNA as templates. The RNA was reverse transcribed and then subjected to 30 cycles of PCR (denaturation at 94°C for 0.5 min, annealing at 50–55°C for 0.5 min, and extension at 72°C for 0.5–1.0 min). Additional information about PCR methodology can be found in **ref. *19***.
2. PCR products of appropriate length are then cloned into a convenient plasmid and sequenced. Candidate PCR products are then labeled and used to screen a cDNA or genomic library to identify a full-length clone for functional studies using high stringency conditions (final wash in 0.1XSSPE and 0.1% SDS) and the methods given in **Subheading 3.1.**

3.3. Database Searching

For more distantly related genes, the human brain and computer algorithms are far more sensitive for identifying meaningful sequence relationships than DNA hybridization techniques. This is how the virally-encoded chemokine receptors have been discovered ***(20–22)***. Since sequencing of the human genome probably will be completed within the next 10 yr, and most of the expressed genes may be identified substantially sooner, screening of candidate DNA identified by database comparisons with known chemokine receptors will become more and more feasible for identifying novel chemokine receptor genes and cDNAs.

The following has been a useful if imperfect set of guidelines for identifying a novel sequence as a reasonably good candidate for a chemokine receptor: length from 340 to 373 amino acids; >25% amino acid identity to known chemokine receptors; multiple acidic residues in the first extracellular segment; a cysteine residue in each of the four extracellular segments; DRYLAIVH motif C-terminal to the third transmembrane domain; and a basic third intracellular loop containing 16 amino acids.

Once a candidate sequence has been cloned, it must then be expressed in a suitable system and tested by gain of function using a chemokine functional

assay, such as radioligand binding, induction of calcium flux, or PI turnover. These functional aspects are covered in detail in other chapters in this volume.

4. Notes

1. Because of the sequence complexity of genomic DNA, isolation of false positive clones by blot hybridization is a significant problem when the stringency of the washing conditions is very low (wash temperature <42°C).
2. PCR cloning using genomic DNA as template assumes that introns are not located within the sequence bounded by the PCR primers. This is the case for most chemokine receptors, however several have introns splitting the ORF in the N-terminal region.
3. Cloning from cDNA or RNA templates assumes the gene of interest is expressed in the tissue source of the RNA.
4. Homology cloning assumes that all chemokine receptors are members of a common family with conserved structure.
5. Positive controls are critical in PCR experiments. Positive controls can include spiking trace amounts of a plasmid for a known receptor into the PCR template. Negative controls can be a reaction mixture minus template.
6. Adjusting the Mg^{2+} concentration can help to improve the sensitivity of PCR reactions.
7. It is simple to tell whether genes related to a known gene exist by analyzing genomic DNA by Southern blot hybridization under low stringency conditions using the ORF of the known gene as probe.

References

1. Murphy, P. M. (1996) Chemokine receptors: cloning strategies. *Methods* **10,** 104–118.
2. Chaudhuri, A., Polyakova, J., Zbrzezna, V., Williams, K., Gulati, S., and Pogo, A. O. (1993) Cloning of the gpD protein cDNA, the major subunit of the Duffy blood group system and the receptor for the *Plasmodium vivax* malaria parasite. *Proc. Natl. Acad. Sci. USA* **90,** 10,793–10,797.
3. Boulay, F., Tardif, M., Brouchon, L., and Vignais, P. (1990) Synthesis and use of a novel N-formyl peptide derivative to isolate a human N-formyl peptide receptor cDNA. *Biochem. Biophys. Res. Commun.* **168,** 1103–1109.
4. Boulay, F., Mery, L., Tardif, M., Brouchon, L., and Vignais, P. (1991) Expression cloning of a receptor for C5a anaphylatoxin on differentiated HL60 cells. *Biochemistry* **30,** 2993–2999.
5. Honda, Z., Nakamura, M., Miki, I., Minami, M., Watanabe, T., Seyama, Y., Okado, H., Toh, H., Ito, K., Miyamoto, T., and Shimizu, T. (1991) Cloning by functional expression of platelet-activating factor receptor from guinea-pig lung. *Nature* **349,** 342–346.

6. Holmes, W. E., Lee, J., Kuang, W.-J., Rice, G. C., and Wood, W. I. (1991) Structure and functional expression of a human interleukin-8 receptor. *Science* **253,** 1278–1280.
7. Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996) HIV1 entry cofactor: functional cDNA cloning of a seventransmembrane, G proteincoupled receptor. *Science* **272,** 872–877.
8. Dobner, T., Wolf, I., Emrich, T., and Lipp, M. (1992) Differentiation-specific expression of a novel G protein-coupled receptor from Burkitt's lymphoma. *Eur. J. Immunol.* **22,** 2795–2799.
9. Birkenbach, M., Josefsen, K., Yalamanchili, R., Lenoir, G., and Kieff, E. (1993) Epstein-Barr virus-induced genes: first lymphocyte-specific G protein-coupled peptide receptors. *J. Virol.* **67,** 2209–2220.
10. Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M-J., Dumont, J. E., and Vassart, G. (1989) Selective amplification and cloning of four new members of the G proteincoupled receptor family. *Science* **244,** 569–572.
11. Gerard, N. P. and Gerard, C. (1991) The chemotactic receptor for human C5a anaphylatoxin. *Nature* **349,** 614–617.
12. Thomas, K. M., Pyun, H. Y., and Navarro, J. (1990) Molecular cloning of the fMetLeuPhe receptor from neutrophils. *J. Biol. Chem.* **265,** 20,061–20,064.
13. Thomas, K. M., Taylor, L., and Navarro, J. (1991) The interleukin-8 receptor is encoded by a neutrophilspecific cDNA clone, F3R. *J. Biol. Chem.* **266,** 14,839–14,841.
14. Murphy, P. M. and Tiffany, H. L. (1991) Cloning of complementary DNA encoding a functional human interleukin-8 receptor. *Science* **253,** 1280–1283.
15. Neote, K., DiGregorio, D., Mak, J. Y., Horuk, R., and Schall, T. J. (1993) Molecular cloning, functional expression, and signaling characteristics of a CC chemokine receptor. *Cell* **72,** 415–425.
16. Murphy, P. M. (1994) The molecular biology of leukocyte chemoattractant receptors. *Annu. Rev. Immunol.* **12,** 593–633.
17. Wells, T. N. and Peitsch, M. C. (1997) The chemokine information source: identification and characterization of novel chemokines using the WorldWideWeb and expressed sequence tag databases. *J. Leukoc. Biol.* **61,** 545–550.
18. Maniatis, T., Fritsch, E. F., and Sambrook, J. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
19. Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J. (eds.) *PCR Protocols, A Guide to Methods and Applications*. (1990) Academic Press, San Diego.
20. Ahuja, S. K., Gao, J.-L., and Murphy, P. M. (1994) Chemokine receptors and molecular mimicry. *Immunol. Today* **15,** 281–287.
21. Lipman, D. J. and Pearson, W. R. (1985) Rapid and sensitive protein similarity searches. *Science* **227,** 1435–1440.
22. Chee, M. S., Satchwell, S. C., Preddie, E., Weston, K. M., and Barrell, B. G. (1990) Human cytomegalovirus encodes three G protein-coupled receptor homologues. *Nature* **344,** 774–777.

10

Generation of Stable Cell Lines Expressing Chemokine Receptors

Christine A. Power and Alexandra Meyer

1. Introduction

Most human and murine leukocytes express multiple chemokine receptors with overlapping ligand specificity. Therefore the creation of stable cell lines expressing specific chemokine receptors greatly facilitates chemokine receptor characterization, particularly in terms of ligand binding specificity, analysis of signal transduction pathways, and for diverse functional assays ranging from chemotaxis to HIV infection. The availability of stable cell lines expressing high levels of chemokine receptors is also becoming increasingly important in high throughput screening approaches for the identification of chemokine receptor antagonists and agonists. The basic steps in the generation of stable cell lines expressing chemokine receptors are as follows:

1.1. Receptor Cloning

The coding sequence of the desired receptor is cloned by standard molecular biology methods ***(1)*** and inserted into an appropriate expression vector containing a selectable drug resistance marker such as neomycin (G418) or zeocin. Currently, the most widely used vector for expression in mammalian cells is pcDNA3 (Invitrogen, San Diego, CA). We use two of its derivatives: pcDNA3.1(+) and pcDNA3.1(+) zeo.

1.2. Transfection of the Vector into the Host Cell Line

The choice of transfection method varies depending on the cell type to be transfected. We generally use calcium phosphate or electroporation to transfect cells. Both methods work well on a large variety of cell types. Cationic

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lipid based transfection systems such as *lipofectamine* (Promega, Madison, WI) can also be used.

Several cell types have been used successfully to generate stable cell lines expressing high levels of receptor. Human embryonic kidney 293 cells (HEK 293) are particularly useful since they can be transfected at high efficiency and have all the components necessary for effective G-protein coupling, which is often a prerequisite for high affinity ligand binding. Other cell lines such as the K562 human erythroleukemia cell line ***(2)*** and L1.2 pre-B-cell line ***(3)*** are also favored. The use of human cell lines for the expression of human chemokine receptors is preferred since some receptors may be incapable of binding their cognate ligands at high affinity due to the absence of appropriate G-proteins in heterologous systems (for example in Chinese hamster ovary [CHO] cells or following transient expression in African green monkey kidney epithelial cell line [COS 7] cells).

1.3. Selection of Stably Transfected Cells

Stably transfected cells are selected within 10–14 d of transfection by growth in medium containing normally lethal concentrations of the appropriate antibiotic.

1.4. Selection of Clones Expressing Functional Chemokine Receptors

Once antibiotic resistant clones have been identified, many strategies can be applied for the analysis of chemokine receptor expression such as:

1. Radio-ligand binding (*see* **Note 1**).
2. Functional assays such as Ca^{2+} mobilization and chemotaxis (*see* Chapters 16 and 11 respectively, in this volume) or microphysiometer analysis ***(4)*** in response to ligand stimulation.
3. Flow cytometric analysis (FACs) using specific monoclonal or polyclonal antibodies (Mabs, Pabs) raised against the chemokine receptor or receptor peptides.

Until recently, highly specific antibodies against chemokine receptors were not generally available. To overcome this problem many groups have successfully employed the use of 9 amino acids from influenza haemagglutinin (HA) inserted at the N-terminal of the receptor, to tag the protein. Monoclonal antibodies against the HA tag are commercially available, (e.g., 12CA5 from Boehringer). While tagged receptors are useful in the absence of specific antibodies, we generally avoid this method as some receptors have altered ligand specificity due to conformational changes induced by the HA tag (C. A. Power, unpublished results).

Below we describe the method currently used in our lab to generate stable cell lines in HEK 293 cells.

2. Materials

All cell culture materials are from Gibco-BRL (Paisley, Scotland) unless stated otherwise.

1. Dulbecco's MEM / Nut Mix F-12 (cat no. 21331-020).
2. Penicillin-Streptomycin (5000U/mL-5000 μg/mL) (cat. no. 15070-022).
3. L-Glutamine 200 m*M* (cat. no. 25030-024).
4. Fetal Bovine Serum (cat. no. 10099-117).
5. Trypsin-EDTA Solution (cat. no. 45300-019).
6. Geneticin (G418) (cat. no. 10131-019).
7. Calcium Phosphate Transfection System (cat. no. 18306-019).
8. Zeocin™ (Invitrogen, cat. no. R250-01).
9. Phosphate-buffered saline (PBS).
10. HEPES buffered saline (HBS) (20 m*M* HEPES, 150 m*M* NaCl; pH 7.3).
11. FACs buffer (PBS containing 1% bovine serum albumin (BSA) and 0.01% sodium azide).
12. Various size tissue-culture dishes (Falcon 6-, 10-, or 15-cm diameter Petri dishes, 24-well cluster and T175 flasks) (Becton Dickinson, Hamburg, Germany).
13. Electroporation cuvets—0.4 cm electrode gap (Bio-Rad cat. no. 165–2088).
14. Stainless steel cloning rings.
15. Autoclaved vacuum grease (Beckman, Fullerton, CA)
16. 37°C, 5% CO_2 humidified incubator.
17. Gene pulser (Bio-Rad).

3. Methods

3.1. Cell Culture

HEK 293 cells are grown in Dulbecco's MEM / Nut Mix F-12 supplemented with 2 m*M* L-glutamine, 100 U/mL penicillin-streptomycin and 10% heat-inactivated fetal bovine serum (FBS; complete medium).

3.2. Transfection

Twenty-four hours before transfection, cell monolayers, grown in a T175 flask, are washed with PBS and treated with 5 mL trypsin-EDTA for 30 s. Trypsin-EDTA is removed and the cells incubated at 37°C for 2 min. The cells are then harvested, resuspended in complete medium (10^6 cells /10 mL), plated in 10 cm diameter Petri dishes and incubated overnight at 37°C in a 5% CO_2 incubator. This normally yields cells at 50% confluency. Three hours before transfection change the medium.

Cells are transfected using the calcium phosphate transfection system as follows: Prepare 1 mL of calcium phosphate—DNA suspension per 10 cm dish of cells. In a polypropylene tube, add 0.5 mL 1X HBS and 10 μL phosphate solution. In a second polypropylene tube, add 20 μg of plasmid DNA (in water)

(*see* **Note 3**) to a volume of 430 µL, add 10 µL of calcium chloride solution, mix gently and add a further 50 µL of calcium chloride solution. Mix gently. Add the contents of the second tube to the first tube, drop-wise, while gently bubbling air through the contents of tube 1. Incubate the suspension at room temperature for 20 min. Mix the precipitate well by pipeting and add it slowly, drop-wise, to the 10 cm plate of cells. Gently swirl the medium in the plate. Incubate at 37°C for 16 h (overnight). The next day remove the medium containing the calcium phosphate precipitate and replace with fresh, complete medium.

If human embryo kidney (HEK) cells are to be transfected by electroporation, proceed as follows: Confluent monolayers of cells are harvested by trypsinization as described above and resuspended in 1X HBS at 2×10^7 cells/mL and kept on ice. Add 30 µg of plasmid DNA to 0.5 mL of cells and transfer to a chilled, 0.4 cm gap electroporation cuvet. Pulse at 960 µF and 260 V. Replace the cuvet on ice for a further 5–10 min then transfer the cells to a 15 cm diameter Petri dish containing 15–20 mL of prewarmed complete medium. Incubate overnight at 37°C. Change the medium the next morning.

3.3. Selection

Twenty-four hours later, remove the medium and add fresh medium containing the appropriate antibiotic. The amount of antibiotic required to kill the nontransfected cells within 7 d should be determined for each cell type used but in our experience for HEK 293 cells, G418 works at 600 µg/mL and zeocin at 100 µg/mL.

Change the medium every 3 days until nonresistant cells are killed off. Resistant clones should be visible after 5 to 7 d.

Pick clones after approx 14 d selection using sterile cloning rings and sterile vacuum grease as follows: Locate the clones that are visible to the naked eye and circle their position on the Petri dish. Remove the medium and wash cells with PBS. Remove PBS. Apply the greased rings over the clones to be picked. Add fresh medium to the plate and 100 µL trypsin-EDTA directly into the cloning rings. Incubate 2 min at 37 C. Remove the trypsin-EDTA solution directly into individual wells of a 24-well plate each containing 2 mL of complete medium with antibiotic. Change the medium every 3 d until the cells are confluent. Then trypsinize as previously and transfer the cells now to 6 cm Petri dishes and subsequently bigger flasks until the desired number of cells is obtained for testing. Once the clones have been selected and propagated, it is advisable to maintain the concentration of antibiotic in the medium (*see* **Note 4**).

Aliquots of the selected clones should at this stage, be frozen down at –80ºC, in complete medium containing 10% DMSO, and then transferred to liquid nitrogen. Any unpicked clones from the master plate may also be stored frozen.

3.4. Selecting Clones with High Level Receptor Expression

We usually pick 10–20 clones of HEK 293 cells and test for high level receptor expression using radio-ligand binding assays or Ca^{2+} mobilization (*see* Chapters 14 and 16 respectively, in this volume) (*see* **Note 2**). If antibodies are available high level receptor expression can be determined by FACS as follows: Remove medium from cells. Incubate with PBS containing 1 m*M* EDTA for 5 min at 37°C. Gently harvest the cells by pipeting and resuspend at 10^6 cells/mL in FACs buffer. Add antireceptor antibody (up to 10 μg/mL) and incubate 60 min at 4°C. As control, cells should be incubated with an isotype control antibody (for MAbs) or with pre-immune serum (for PAbs). Wash cells 3× in FACS buffer. Resuspend cells in 200 μL FACS buffer containing FITC or PE -labeled 2nd antibody and incubate for 30 min at 4°C. Wash cells 3× in FACS buffer and finally resuspend in 200 μL FACS buffer. Fluorescence intensity is measured by flow cytometry.

4. Notes

1. This may require custom synthesis as there are only a limited number of radio-ligands commercially available (from NEN or Amersham-Pharmacia)
2. One of the problems encountered with stable cell lines has been the loss of receptor expression as a result of long term maintenance of cells in culture. This can usually be avoided by freezing down large numbers of early passage number cells which can be revived as and when required.
3. A mock transfection with vector without insert acts as a suitable negative control for subsequent assays.
4. When generating stable cell lines in nonadherent cells such as L1.2, after 10–14 d of antibiotic selection, resistant cells are usually plated as single cells in 96-well plates (determined either empirically by limiting dilution or by FACs sorting). Individual clones can then be grown up and tested. Alternatively, when antibodies are available, the resistant cells can be analyzed for antibody binding by FACs and the positive cells gated. Single cell sorting is still required however to ensure that the population is homogeneous.

References

1. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. Baba, M., Imai, T., Nishimura, M., Takagi, S., Hieshima, K., Nomiyama, H., and Yoshie, O. (1997) Identification of CCR6, the specific receptor for a novel lymphocyte-directed CC chemokine, LARC. *J. Biol. Chem.* **272,** 14,893–14,898.
3. Ponath, P. D., Qin, S., Ringler, D. J., Clark-Lewis, I., Wang, N., Kassam, H., Shi, X., Gonzalo, J-A., and Newman, W. (1996) Cloning of a human eosinophil chemoattractant, eotaxin, expression, receptor binding and functional propreties

provide a mechanism for selective recruitment of eosinophils. *J. Clin. Invest.* **97,** 604–612.

4. Pitchford, S., Hirst, M., Wada, H. G., Chan, S. D. H., Timmermans, V., and Humphries, G. M. K. (1997) Characterization of functional activity of chemokine receptors using the cytosensor microphysiometer. *Methods Enzymol.* **288,** 84–108.

11

Modified Microchemotaxis Assays

Dennis D. Taub

1. Introduction

Chemotaxis is the process by which leukocytes are directed to sites of inflammation under the influence of a concentration gradient of the soluble chemotactic molecules. Upon encountering a chemotactic molecule, responding leukocytes begin to migrate directionally from regions of low ligand concentrations toward the sites of chemoattractant production that typically possess more substantial levels of soluble chemotactic factors. A number of endogenous proteins have been shown to mediate leukocyte migration, including activated serum components, platelet activating factor (PAF), eucosinoids, cytokines (e.g., IL-1, TNFα, IFN-γ), and neuroendocrine hormones ***(1)***. Although many of these factors are believed to play some role in inflammation, their relevance to leukocyte infiltration into inflammatory sites or homing to lymphoid organs has been brought into question.

Over the past ten years, more than 40 unique human and murine cytokines have been identified as members of "chemokine" superfamily of chemoattractants. These cytokines have been shown to induce the directional migration of selected cell types including neutrophils, monocytes, macrophages, dendritic cells, lymphocytes, basophils, eosinophils, and fibroblasts.

Based on the presence or absence of conserved cysteine residues within their primary sequence, the chemokine superfamily can be separated into four distinct subfamilies called the C-X-C (or α), the C-C (or β), the C, or the C-X-X-X-C subfamilies ***(1–2)***. Chemokines share many other similarities including their high basic nature as well as their ability to bind heparin through heparinbinding domains. These molecules exhibit sequence identity at the amino acid level between 24 and 80%. Historically, the chemokine subfamilies

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were biologically distinguished from one another by their apparent leukocyte specificity in mediating cell migration ***(1)***. The early rule was that C-X-C chemokines (in particular, those chemokines containing an ELR motif in their amino terminal sequence) induce neutrophil but not monocyte migration while C-C chemokines predominantly act on monocytes and macrophages with no activity on neutrophils. However, with the examination of additional leukocyte subsets and the discovery of additional chemokine subfamily members, distinguishing chemokine subfamilies based on leukocyte motility and specificity has become invalid. Today, we know that, besides neutrophils and monocytes, both C-X-C and C-C family members are active on a number of cell types, including basophils, eosinophils, lymphocytes, endothelial cells, epithelial cells, melanocytes, smooth muscle cells, keratinocytes, and hepatocytes. Several but not all C-C chemokines have also been found to induce eosinophil, basophil, and mast cell migration ***(1–2)***. All of the C-C and C chemokines as well as several C-X-C chemokines have been shown to induce human lymphocyte migration both in vitro and in vivo ***(1–2)***. Furthermore, contrary to the early rule, several C-C chemokines, including MIP-1α and MCP-3, are capable of stimulating neutrophil migration and activation ***(1–2)*** while C-X-C chemokines, like IP-10 and MIG, are modest monocyte chemoattractants ***(1–2)***.

While the biological effects of chemokines on mononuclear and granulocytic cell populations can be measured using a number of biological and biochemical assays (as described within this volume), the Boyden chamber microchemotaxis assay has been classically utilized to assess the ability of chemokines to facilitate cell movement ***(3)***. For the past 25 yr, both monocyte and neutrophil migration has been assessed using a Boyden chamber apparatus. This chamber permits the placement of cells to an upper chamber with a filter separating them from the chemoattractants placed within the lower chamber. Using polycarbonate filters precoated with extracellular matrix proteins, the directional migration of lymphocytes can also be assessed using a modified assay of chemotaxis ***(4–6)***. After incubation, the number of migrant adherent cells on the lower surface of the filter are counted using light microscopy or an image analyzer.

2. Materials

2.1. Microchemotaxis Assay Using Polycarbonate Filters

1. Recombinant human or murine chemokines are available from a number of different companies. Examples of such chemokine reagents: Recombinant human or mouse MIP-1α (R&D Systems #270-LD, #450-MA), MIP-1β (R&D Systems #451-MB, #271-BM), RANTES (R&D Systems # 278-RN), MCP-1 (R&D Systems #279-MC), IL-8 (R&D Systems #208-IL, Minneapolis, MN), or IP-10 (PeproTech #300-12, Rocky Hill, NJ).

2. Chemotaxis medium: RPMI 1640 containing 25 m*M* HEPES and either 0.5% heat-inactivated fetal calf serum (for lymphocyte chemotaxis) or 1% bovine serum albumin (BSA Fraction V, Sigma #A9306) (for monocyte and granulocyte chemotaxis).
3. Multiwell chemotaxis chamber and an accessory pack containing clips and a wiper blade (Neuro Probe #AP48 and #P48AP, respectively).
4. Polycarbonate membranes with 5-μm pores (Neuro Probe #NFA3) with or without polyvinylpyrrolidone (PVP) coating.
5. Extracellular matrix proteins: Human plasma fibronectin (Gibco/BRL, Gaithersburg, MD # 6803016IC), mouse collagen type I or IV (Gibco/BRL # 6803018IC), mouse laminin (Gibco/BRL #680-3017IC).
6. Matrixcoated filters for the assessment of lymphocyte migration ***(4–6)***.
   a. Prepare a solution of extracellular matrix at a concentration of 20 μg/mL in H_2O. Various lots of matrix proteins (at least 3–5 lots) should be prescreened for optimal cell reactivity.
   b. Float either the shiny or the dull side of the uncoated polycarbonate filters in 10mL of a matrix solution in a Petri dish for 1 h at 37°C or overnight at 4°C to coat predominantly one side of the filter. Filters coated on both sides also permit T-cell movement; however, these membranes permit greater T-cell adhesion to the upper surface and may result in a false positive impression that migration has occurred.
   c. Prior to use, extensively rinse the filters in phosphate-buffered saline (PBS) and air-dry in a laminar flow hood. Matrix-coated filters should be prepared the same day as the chemotaxis assay to assure the matrix integrity.
7. Cell suspension: Purified leukocytes or leukocyte subsets.
8. DiffQuik stain (Baxter, Muskegon, MI #B4132-1).
9. Coverslips.
10. Glass slides.
11. Zeiss Axioskop microscope (model D-7082 Zeiss; Oberkochen, West Germany) using a video camera (Ikegami Electronics, Maywood, NJ).
12. Optomax image analyzer (Model V from Optomax, Hollis, NH).

2.2. Microchemotaxis Assay using Nitrocellulose Filters

Similar reagents are utilized for chemotaxis on the thicker nitrocellulose filters as with polycarbonate filters with the following exceptions:

1. Thick nitrocellulose filter membrane with 3–5 μm diameter pores (Neuro Probe, Cabin John, MD). As with polycarbonate filters, the thicker nitrocellulose filters may also be coated with extracellular matrix proteins to facilitate lymphocyte migration (*see* **Subheading 2.1.**).
2. 18.5% formalin (Aldrich, Milwaukee, WI).
3. Mayer's hematoxylin (Sigma Chemical Co.).
4. Propanol (Aldrich).
5. Xylene (Aldrich).

3. Methods

3.1. Leukocyte Chemotaxis Using Polycarbonate Filters

1. To assay a chemokine ligand for migratory activity, prepare a series of dilutions of the chemokine to be tested in chemotaxis medium. Place in triplicate in the bottom wells of the multi-well microchemotaxis chamber. Typically, 24- and 48-well microchemotaxis chambers are available for these assays.
   a. Include a set of wells containing chemotaxis medium alone as a negative control. If available, a series of wells should also be set up with a known positive chemoattractant as a positive control.
   b. For optimal leukocyte migration, polycarbonate filters with a pore-diameter of 3-μm to 5-μm should be utilized. Pore sizes of greater diameters may lead to significantly greater background responses. Larger pore sizes may be utilized when examining chemokine-induced migration of tumor cells or various stromal cell populations.
   c. Depending on the microchemotaxis chamber, the volume in the bottom wells can hold between 25–28 μL of fluid (depending on the chemotaxis chamber). Caution should be used in adding the chemokine dilution to the wells as excessive fluid may lead to cross-contamination between wells upon chamber assembly. The added volume should be slightly convex over the rim of the well. Too little liquid leads to bubble formation which interferes with cell migration in the filter.
   d. The following are examples of positive chemokine controls for various leukocyte subsets. For T-cell migration, the C-C chemokines, MIP-1α, MIP-1β, RANTES, MCP-1, MCP-3, MIP-3, and Exodus-1, and the lymphotactin, have been shown to be potent T-cell chemotactic agents which induce optimal migratory activity between 0–1 and 10 μM ***(4–6)***. For monocytes and dendritic cells, the C-C chemokines, MIP-1α, MIP-1β, RANTES, MCP-1, MCP-3 and MCP-3α, have been shown to induce optimal chemotaxis at concentrations ranging between 3–30 μM ***(1–2,5)***. For neutrophils, the CXC chemokines, IL-8, MIP-2, NAP-2, ENA-78, and GCP-2, have been shown to induce optimal chemotaxis at concentrations ranging between 3–30 μM ***(1–2,5)***.
2. Using gloves and forceps, place the polycarbonate filter over the chemokine- or medium-filled wells of the chamber. Adjust the position of the filter if the peripheral wells are not covered. Over adjustment of the filter may result in cross-contamination of the wells in the chamber. The chamber gasket and lid should be assembled quickly after the placement of the filter. Pressure should be applied to the center and the corners of the chemotaxis chamber lid prior to final chamber assembly to prevent cross-contamination and bubble formation.
   a. When utilizing polycarbonate filters, one should use polyvinylpyrrolidone (PVP)-coated filters for monocytes and dendritic cells and PVP-free (PVPF) for neutrophils and granulocytes ***(5)***. For lymphocyte chemotaxis, optimal migration may be observed on matrix-coated filters (as described in **Subheading 2.1.**).

   b. Polycarbonate filters possess a dull and a shiny side. When placing a filter on a chemotaxis chamber, one should place the shiny side up for optimal chemotaxis. Also, cut or make a notch on one of the corners of the filter so that the orientation of the filter can later be assessed post staining.
3. Dilute the purified cell suspension to the concentration of 1×10^6 cells/mL in chemotaxis medium. For 48-well microchemotaxis chambers, 2.5 mL of cell suspension will be required for each chamber. Add 50 μL of the cell suspension to each of the upper wells of the chemotaxis chamber. Once added, incubate in a 37°C humidified-air CO_2 chamber.
   a. Cell concentrations ranging from 10^5 to 10^7 cells/mL may be utilized in the chemotaxis assays. However, utilizing a more concentrated cell suspension ($\geq 10^6$ cells/mL), more reproducible chemotactic responses will be observed.
4. After incubation, invert the chamber to decant residual media and cells from the top wells of the chamber. Remove the nuts from the chamber and grasp both ends of the filter with the special clamps supplied by the manufacturer. Wash the undersurface of the filter (where nonmigrating cells attach) gently in PBS. Gently scrape the undersurface of the filter by passing it 3–4 times over the rubber blade supplied by the manufacturer.
   a. Under certain circumstances, migrating leukocytes may fall from the filter into the bottom wells of the chamber. Thus, it may be advantageous to count these cells just in case the filters exhibit poor results.
5. Air-dry the filter for 15 min. After drying, stain filters with Diff-Quik according to the manufacturer's directions. Filters are then dried, mounted in oil on a slide, and then covered with a cover slip. Migrating T cells are counted by selecting how many random fields for each well with a 40× objective on a standard microscope or a microscope connected to a video camera and an image analyzer.
   a. The location of the wells of the filter should be marked with a marker while on the slides after mounting. As mentioned above, the "notching" of the filters will greatly assist in orienting the filters and marking which well contains which chemokine. It is essential to code the slides and to present them as unknowns to the counter.
   b. Counting 5 to 10 random high power fields per filter yield more reproducible and representative results. The results can be expressed as either the mean number of migrating cells per high power field or as a percentage of the total cells that have migrated.
   c. Caution should be used in counting the filters as adherent cells not scraped off the top surface of the filters may be mistaken for migrant cells. By focusing on the pores, adherent can be easily distinguished from migrating cells.
   d. The migrating cells are frequently adherent to one another and leave the appearance of grape clusters. These are counted as one cell resulting in an under estimate of responses (unless one is able to assess the exact number of cells within such a clump).

6. Express the results in the following ways:
   a. Mean (± SD) number of migrating cells in 5 to 10 high-power fields tested in triplicate. These are cells that have completely migrated through the filter.
   b. Chemotactic index as calculated below:
      C.I. = Migrating cells to test reagent/migrating cells to medium
7. Statistical analysis for these assays can be performed using many statistical programs including a twotailed student t-test or a KruskalWallis nonparametric analysis of variance on averaged triplicate values.
   a. A significant difference in overall migration between two conditions required that significant differences in cell counts ($p < 0.05$) be observed within 5 high-power fields in triplicate wells.

3.2. Leukocyte Chemotaxis: Nitrocellulose Filters

Some laboratories utilize thicker nitrocellulose filters rather than polycarbonate filters to assess chemotaxis. The main advantage of the nitrocellulose filters is that the precise distance a cell migrates in response to a given chemokine may be quantitated. Overall, chemotaxis using the nitrocellulose filters is almost exactly the same as with the polycarbonate filters with the exception of how the filters are treated after incubation. These differences are described below:

1. After incubation, invert the chamber to decant residual media and cells from the top wells of the chamber. Remove the nuts from the chamber and grasp both ends of the nitrocellulose filter with the special clamps supplied by the manufacturer. Wash the undersurface of the filter (where nonmigrating cells attach) gently in PBS. Gently scrape the undersurface of the filter by passing it 3–4 times over the rubber blade supplied by the manufacturer scraped rinsed in PBS, and then fixed with 18.5% formalin.
2. The washed filter is then stained with Mayer's hematoxylin, cleared with propanol, subsequently treated with xylene.
3. The membranes are then placed on glass slides with immersion oil after which glass cover slips are applied.
4. Chemotaxis analysis was performed using a Zeiss Axioskop microscope using a video camera and an Optomax image analyzer. Beginning at the top of the filter, the number of cells per 200× high-powered field was measured at successive 10 μm intervals down through the nitrocellulose filter. Each well ("spot") on the filter was measured in three random areas and each sample was tested in triplicate wells. Video gain and Optomax counting parameters were determined once for each filter and remained unchanged for reading that filter.
5. The results are expressed as the number of cells per high power field for each 10 μm distance. Statistical analysis for thick filter assays can be performed using many methods including a twotailed student to test or a KruskalWallis nonparametric analysis of variance on averaged triplicate values.

a. a significant difference in overall migration between two conditions required that significant differences in cell counts ($p < 0.05$) be seen at 3 of 4 consecutive depths.

4. Notes

1. Chemotaxis assays provide a very sensitive indicator of cellular mobilization and can easily be mastered by anyone with average laboratory experience. Several critical steps must be followed in loading a chemotaxis chamber. The first is to add the correct volume of chemokine to the bottom wells of the chamber. Typically, 26 μL is the correct volume to be loaded. Too small a volume results in air bubbles in the wells, while too large a volume overflows the wells.
2. Another critical step is the placement of the filter to the filled wells of the chamber. Caution should be utilized when placing the filter on the filled wells as excessive movement may result in contamination between the wells. Furthermore, in lymphocyte migration assays, matrix-coated filters should be coated at least 2 h prior to performing the assay. The filter should be extensively washed and thoroughly dried prior to the placement of the filter in the chamber. A wet filter may permit leaking between the wells of the chamber. In addition, nonbound extracellular matrix proteins must be removed by extensive washing as residual matrix may result in a loss of adhesion to the filter.
3. Different cell concentrations can also be utilized in the chemotaxis assay. Typically, a more concentrated cell suspension (10^6–10^7 cells/mL) yields better cell migration. However, cell concentrations exceeding 2×10^7 cells/mL and less than generally 5×10^4 cells/mL may result in variable T-cell movement or meager migration. Chambers should be incubated at 37°C for at least 3 h to generate a T-cell migratory response. Incubation times of greater than 4 h generally result in a decrease in the number of T cells adherent to the chemotaxis filter, probably due to detached T cells falling off the filter into the well of the chamber. It should be noted that it requires 4 h for the chemokines to reach total equilibrium between the top and bottom wells of the chamber in the presence of T cells. Thus, incubation times greater than 4 h will not enhance a T-cell migratory response.
5. The activation state of a cell population may also dramatically affect the chemotactic response. While freshly isolated cells (depending on the cell population) migrate effectively in response to chemokines, activation of leukocytes with various growth factors or activation stimuli (e.g., immobilized anti-CD3 antibody for lymphocytes) yield greater migratory response. However, overactivated or rapidly dividing cells fail to reproducibly migrate in response to chemokines as they tend to adhere to the top of the filters. In addition, chemokine receptor expression can be greatly influenced by the activation state of the leukocyte population being examined.
6. Various lots of extracellular matrix proteins should be screened for use in chemotaxis experiments. Active preparations of collagen type IV, laminin, and

fibronectin are not common and may require screening many lots from many different companies. Once an active lot is identified, large quantities should be purchased and stored for future use.

7. In examining human leukocyte responses to chemokines, donor-to-donor variation should be expected. It is not uncommon, in particular with lymphocytes, to find that 1 out of 10 donors may fail to migrate in response to any chemotactic stimuli.
8. Once chemokines are diluted, the wells of the chemotaxis chamber can be loaded and sealed within 5–10 min. While the chamber incubation time varies depending on the leukocyte population being examined, most cells (excluding lymphocytes which require up to 4 h) will yield significant migration within 90 min of the initiation of the assay. Shorter incubation times can be utilized, however, this will result in a diminished migratory response. In addition, filter staining and drying will require approx 30 min. Counting migrant cells can be performed manually on a light microscope or by an image analyzer. Manually, one should allow up to 1 h to count the 48 stained wells of a single chemotaxis filter.
9. Newer migration chambers are available which permit the assessment of chemotaxis in a 96-well format using flourescently labeled cells. These special chambers (NeuroProbe) allow the placement of darkened 96-well plates within the apparatus. In these chambers, plate-size polycarbonate filters are utilized to separate the upper wells from the dilutions of chemokines placed in the lower wells. Once the assay is finished, EDTA is added to the upper wells to disengage the migrating fluorescent cells from the filter so the total florescence on the plate can be assessed using a microflorimeter. This newer method permits the rapid qualitative assessment of cell migration.

References

1. Taub, D. D. (1996) Chemokines-Leukocyte Interactions: The Voodoo That They Do So Well. *Cytokine and Growth Factor Reviews* **7(4),** 355–376.
2. Baggiolini, M., Dewald, B., and Moser, B. (1994) Interleukin-8 and related chemotactic cytokines—CXC and CC Chemokines. *Adv. Immunol.* **55,** 97.
3. Falk, W. R., Goodwin, R. H., Jr., and Leonard, E. J. (1980) A 48-well microchemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J. Immumol. Methods* **135,** 239–247.
4. Taub, D. D., Clark, D., and Turcovski-Corrales, S. M. (1995) Chemotaxis of T lymphocytes on extracellular matrix protein: analysis of the *in vitro* method to quantitate chemotaxis of human T cells. *J. Immunol. Meth.* **184,** 187–198.
5. Taub, D. D. (1995) The α and β chemokine superfamily. *Current Protocols in Immunology* (ed. John E. Coligan, et al.) Unit 6.12, John Wiley and Sons, New York, NY.
6. Taub, D. D., Conlon, K., Lloyd, A. R., Oppenheim, J. J., and Kelvin, D. J. (1993) Preferential migration of activated $CD4^+$ and $CD4^+$ T cells in response to MIP-1" and MIP-1α. *Science* **260,** 355–358.

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Transwell Chemotaxis

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1. Introduction

Numerous and diverse biological functions are regulated by chemokines. In addition to the well characterized proinflammatory activities such as integrin activation, chemotaxis, lipid mediator biosynthesis, superoxide radical production, and granule enzyme release (reviewed in **refs. *1–4)***, chemokines have been shown to suppress and stimulate angiogenesis ***(5–7)***, suppress hematopoiesis ***(8–10)***, suppress apoptosis ***(11)***, control viral infection ***(12,13)***, and effect leukocyte differentiation ***(14)***. Among the proinflammatory activities, chemotaxis in particular has received considerable attention as a target for novel antiinflammatory therapeutics (reviewed in **ref. *15***).

Chemokines mediate effector functions by binding to seven transmembrane spanning G-protein coupled receptors (reviewed in **ref. *16***). Most of the proinflammatory activities have been shown to be pertussis toxin sensitive indicating that the receptors are coupled to $G_{\alpha i}$ subunits. Consistent with these data, chemokine receptor mediated chemotaxis has been shown to be dependent on activation of the $G_{\alpha i}$ subunit and subsequent release of free $G\beta\gamma$ dimers, with the free $\beta\gamma$ subunit responsible for initiating signal transduction leading to cell migration ***(17,18)***. Although capable of coupling to a variety of G_{α} subunits in lymphocytes, including $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha 16}$, it remains unclear how $G_{\alpha i}$ selectively mediates the chemotactic response as $G_{\alpha q}$ and $G_{\alpha 16}$ activation also release free $\beta\gamma$ subunits. Even less well understood are the signaling pathways associated hematopoetic, apoptotic, and leukocyte differentiation activities.

Directed cell migration mediated by chemokine-chemokine receptor interactions involves several physiological changes in the cell including actin polymerization and shape change, integrin activation and recycling, and chemokine receptor internalization and recycling. These actions promote the redistribu-

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tion of integrins and chemokine receptors leading to cell polarization with the formation of lamellipodia at the leading edge and uropod formation at the trailing. In fact, redistribution of chemokine receptors was recently demonstrated with antibodies to CCR2 and CCR5 where these receptors were shown to polarize to the leading edge during T-cell migration ***(19)***.

Several types of chemotaxis assays have been described including Boyden chamber, migration under agarose, and transwell chemotaxis. Transwell chemotaxis provides for much higher throughput with systems developed to accommodate 24-, 48- and 96-well formats. Although requiring larger amounts of chemokine and greater numbers of cells, we prefer the 24-well format over the 48- and 96-well because of better well-to-well and plate-to-plate consistency with duplicate wells being sufficient for most studies. Also, the larger starting cell number is not only amenable to our preferred counting method but sufficient cells are recruited to allow for various down stream processing such as phenotypic analysis by flow cytometry when starting with mixed cell populations.

Transfectants generally have very low background migration and the method described below can be easily optimized for receptors expressed in most common laboratory cell lines. However, if primary cells or purified subpopulations of primary cells are being assayed each must be optimized with respect to the following parameters to obtain an optimal signal-to-noise ratio:

1. Number of cells per well;
2. Membrane filter type, such as polycarbonate, polyester, nitrocellulose, etc.;
3. Membrane pore size/density, generally 1.0–8.0 μm, with smaller pore size filters having greater pore density;
4. Bare filter vs coated with fibronectin, collagen, endothelial cell monolayer, etc.; and
5. Incubation time, which can range from 30 min to 4 h.

2. Materials

1. Chemokine receptor transfectant and appropriate growth media.
2. RPMI-1640 (Gibco-BRL,).
3. M199 (Gibco-BRL).
4. Bovine Serum Albumin (BSA, Sigma Cat. No. A7888).
5. Fetal Calf Serum (FCS, Gibco-BRL).
6. Transwell® tissue-culture inserts/plates (6.5 mm diameter, 3 μm pore size) Costar, Cambridge, MA, Cat. No. 3415.
7. Tissue-culture incubator.
8. Chemokines (Peprotech, R&D Systems).
9. Endothelial cell line (e.g., ECV304; ECACC).
10. Sterile, endotoxin free, tissue culture-grade water.
11. Phosphate-buffered saline (PBS) without Mg^{2+} Ca^{2+} (PBS, Gibco-BRL).

12. Histopaque 1077 (Sigma).
13. Anti-CD3 antibody (ATCC).

3. Methods

Chemokine stock solutions may be prepared prior to the assay by resuspending lyophylized chemokine in sterile, endotoxin free water or PBS at about 1 mg/mL. Although most chemokines can withstand a few freeze-thaw cycles without substantial loss of activity, this should be avoided by aliquoting the stock solutions in a convenient volume and storing at –80°C until use. Once aliquots have been thawed, most chemokines will remain stable at 4°C for several days to a few weeks.

We generally assess the protein concentration of new chemokine stock solutions by OD_{280} using the appropriate extinction coefficient for each chemokine or by Bradford assay. Although this is not often practical when very small quantities of chemokine are purchased because 10–20% of the material may be sacrificed for an accurate determination, we have found that the actual protein concentration may vary by as much as 20–40% between lots which can significantly effect the reproducibility of chemotaxis assays.

3.1. Transwell Chemotaxis of Chemokine Receptor Transfectants

The assay described in **steps 1–11** provides a general method for determining the chemotactic dose response of a chemokine receptor transfectant to a ligand. With minimal optimization this method is suitable for assaying stable receptor transfectants established in several commonly used and available human and murine cell lines of hematopoeitic origin. In fact, this method may be used to assess the chemotactic potential of untransfected cell lines as well. Using this method we routinely achieve signal-to-noise ratios of 200–500 using chemokine concentrations giving 50% of maximal response.

1. The day before the assay, receptor transfectants should be split back to exponential phase growth, $0.25–0.5 \times 10^5$ cells per mL, in appropriate cell culture media such as RPMI-1640 plus 10% FCS.

   NOTE: If cells are maintained in selective media to ensure retension of a transfected plasmid, it may be necessary to remove the selective agent at this time. Although we routinely use G418 selection and do not find any effect on chemotaxis, other selection agents may interefer with the assay. Mycophenolic acid, for example, has been determined to effect migration of chemokine receptor transfectants established in some cell lines.
2. On the day of the assay prepare 1.5 mL of each chemokine dilution to be assessed by diluting the chemokine stock solution in chemotaxis media (chemotaxis media is RPMI-1640, 0.5% BSA) prewarmed to 37°C.

NOTE: Depending on the chemokine-receptor pair, chemotactic activity may be detected at concentrations as low as 0.1 n*M* ligand or lower and peak activity may be seen as high as 100 n*M* or higher. Five, 10-fold dilutions starting with 0.1 n*M* will generally cover the physiologically relevant active range of almost all chemokines. A 1 μ*M* assay point in duplicate, however, will use a substantial amount of chemokine in the 24-well assay and most physiologically relevant chemokines are on the "post-peak" side of the bell-shaped curve at 500 n*M*.

3. Prepare the chemotaxis plate by adding 600 μL of appropriate chemokine dilution to the bottom wells which either do not contain transwell inserts or have had the transwell inserts removed to another 24-well plate. Each point is performed in duplicate and two wells should be designated as negative controls and receive 600 μL of chemotaxis media only. These wells are used to determine the assay background. The plate may be placed at 37°C, 5% CO_2 to maintain temperature while preparing cells.
4. Count receptor transfectants and harvest the appropriate number of cells for the assay using 1×10^6 cells per well.
5. Wash the cells once in chemotaxis media prewarmed to 37°C and resuspend at 1×10^7 cells per mL in chemotaxis media.
6. Dispense 100 μL of cell suspension into the transwell inserts.
7. Gently transfer the transwell inserts containing cells into the wells containing the previously dispensed chemokine or control media. It may be helpful to set the inserts into the bottom wells at an angle to avoid trapping air bubbles between the transwell insert membrane and the media in the bottom well.
8. Gently place the 24-well plate in a 37°C, 5% CO_2 incubator and allow the assay to run undisturbed for 3–5 h.
9. After removing the chemotaxis plates from the incubator, remove the transwell inserts from the bottom wells and discard.
10. Gently resuspend the cells which have migrated through the membrane and settled in the bottom well.
11. Transfer a 500 μL aliquot to another tube and count cells.

3.2. Transwell Chemotaxis of CD3 Blasts Using an Endothelial Cell Line

As discussed in the Introduction developing a robust 24-well bare filter assay using primary leukocytes may require substantial optimization of various parameters depending on the cell type. The protocol described in **Subheading 3.1., steps 1–11** may be used as a basis on which various parameters are optimized. However, use of an endothelial cell line monolayer, such as ECV304, grown on the transwell membrane provides a substantial barrier to background migration and will allow essentially the same protocol to be used with minor modifications for several leukocyte types including granulocytes, monocyte, and activated T cells.

Use of an endothelial cell line should not be considered a substitute for primary endothelial cells such as HUVEC, as different endothelial cell lines may vary in the type and level of adhesion molecules expressed as well as their response to various stimuli such as cytokines. Endothelial cell lines provide a convenient, homogenous, and defined (if not necessarily relevant) cell monolayer through which leukocytes must migrate in the chemotaxis assay. We find that use of an endothelial cell line to reduce the background migration of primary cells not only improves the signal-to-noise ratio of the assay but also the reproducibility of the assay as compared to HUVEC.

3.2.1. Production of CD3 Blasts

3.2.1.1. PBMC Isolation

1. Collect heparinized blood.
2. Dilute 1 : 2 with room temperature PBS and place 30 mL of blood/PBS mixture in a 50 mL conical tube.
3. Gently layer 15 mL histopaque solution beneath the blood/PBS mixture using a 10 mL pipet.
4. Centrifuge 25 min in a horizontal rotor at 900*g* at room temperature. Granulocytes and erythrocytes will pellet to the bottom of the tube with the peripheral blood mononuclear cell (PBMC) forming a layer at the interface between the histopaque and blood plasma.
5. Remove the upper layer containing plasma and platelets and discard. Transfer the mononuclear cell layer to another tube and wash cells twice with PBS plus 2% FCS or RPMI plus 2% FCS to remove remaining platelets.
6. Resuspend the PBMC at 4×10^6 mL in RPMI 1640 + 10% FCS.

3.2.1.2. Stimulation

1. Coat wells of a 24-well plate with an anti-CD3 antibody such as OKT3 (ATCC) by diluting the antibody to a final concentration of 1–10 μg/mL in PBS and dispensing 0.5 mL per well.
2. Incubate the plate at 37°C for at least 1 h and remove the remaining antibody solution.
3. Wash the coated wells 4 times with 2 mL of PBS.
4. After the last wash add 0.5 mL of RPMI 1640 + 10% FCS to each well.
5. Add 0.5 mL of PBMC to each of the coated wells.
6. Incubate the plate at 37°C, 5% CO_2.
7. Observe the plate on day 3. Cells are ready for transfer when large clusters can easily be seen.
8. When the cells are ready to be transfered to a flask, gently resuspend the cells by pipeting and transfer to a 15-mL conical tube.
9. Spin cells at room temperature for 5 min at 200*g*.
10. Resuspend cells in RPMI 1640 + 10% FCS supplemented with 150 U/mL of IL-2.

11. Incubate the cells at 37°C, 5% CO_2.
12. Monitor the cells daily. During the first week the cells will grow rapidly.

3.2.2. Preparation of Transwell Inserts with Endothelial Cell Monolayer

1. Count endothelial cells (ECV304) and resuspend at 2×10^6 cells/mL in growth media, M199 plus 10% FCS.
2. Dispense 600 μL of growth media into the bottom well of the chemotaxis plate.
3. Dispense 100 μL (2×10^5 cells) into the transwell inserts.
4. Gently transfer the transwell inserts into the bottom wells containing growth media. It may be helpful to transfer the inserts at an angle to avoid trapping air bubbles between the transwell insert membrane and the media in the bottom well.
5. Incubate the plates at 37°C, 5% CO_2 for at least 24 h to allow the monolayer to form. Plates may be used for up to two weeks.

3.2.3. Chemotaxis Assay

1. Prepare 1.5 mL of each chemokine dilution to be assessed by diluting the chemokine stock solution in chemotaxis media (chemotaxis media is a 50:50 mixture of endothelial cell growth media without FCS and RPMI-1640, plus 0.5% BSA) prewarmed to 37°C.

   NOTE: Although a dose response can be seen with leukocytes assayed using an endothelial cell line, a bell-shaped curve is usually not observed with concentrations up to 1 μ*M* if the monolayer forms a tight seal on the membrane.
2. Set up the chemotaxis plate by adding 600 μL of appropriate chemokine dilution to the bottom wells of a 24-well plate which do not contain transwell inserts. Each point is performed in duplicate. Two wells should be designated as negative controls and receive 600 μL of chemotaxis media only. These wells are used to determine the assay background. The plate may be placed at 37°C, 5% CO_2 to maintain temperature while preparing inserts.
3. Carefully, remove the culture supernatant from the transwell inserts containing the endothelial cell monolayer. Do not touch or otherwise disturb the cells.
4. Gently dispense 100 μL of CD3 blasts into the endothelial monolayer coated transwell inserts.
5. Transfer the inserts containing CD3 blasts to the bottom wells of the chemotaxis plate at an angle to avoid trapping air bubbles.
6. Incubate the plates at 37°C, 5% CO_2 undisturbed for 0.5–1.0 h.
7. After removing the chemotaxis plates from the incubator, remove the transwell inserts from the bottom wells and discard.
8. Gently resuspend the cells which have migrated through the membrane and settled in the bottom of the well.
9. Transfer a 500 μL aliquot to another tube and count cells.

There may be a substantial amount of endothelial cell contamination and debris in the bottom well which can make it difficult to quantitate CD3 blasts

by direct counting. Blasts may be labeled with BCECF (Molecular Probes, Eugene OR) prior to running the assay by incubating the cells for 30 min at 37°C in chemotaxis media containing 0.5 mg/mL BCECF followed by 2 washes in chemotaxis media. This will allow the migrated blasts to be distinguished from the endothelial cells and counted under a fluorecent microscope.

We prefer to avoid labeling cells prior to assay for convenience as well as the fact that certain agents may interfer with some modifications of the assay as well as downstream use of the cells. We have also found that BCECF can leak from labeled cells and be taken up by the endothelial cells which reduces the signal-to-noise ratio. Alternatively, we prefer to count cells by flow cytometry. CD3 blasts (and other leukocyte types) are easily distinguishable from endothelial cells using forward vs side scatter. Simply gate on the CD3 blasts and count each sample for a specific length of time, typically 30 s. This method is quite reproducible and correlates well with other counting methods ***(20–22)***.

References

1. Oppenheim, J. J., Zachariae, C. O., Mukaida, N., and Matsushima, K. (1991) Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu. Rev. Immunol.* **9,** 617–648.
2. Schall, T. J. and Bacon, K. B. (1994) Chemokines, leukocyte trafficking, and inflammation. *Curr. Opin. Immunol.* **6,** 865–873.
3. Baggiolini, M. and Dahinden, C. A. (1994) CC chemokines in allergic inflammation. *Immunol. Today* **15(3),** 127–133.
4. Bacon, K. B. and Schall, T. J. (1996) Chemokines as mediators of allergic inflammation. *Int. Arch. Allergy Immunol.* **109(2),** 97–109.
5. Cao, Y., Chen, C., Weatherbee, J. A., Tsang, M., and Folkman, J. (1995) gro-beta, a -C-X-C- chemokine, is an angiogenesis inhibitor that suppresses the growth of Lewis lung carcinoma in mice. *J. Exp. Med.* **182,** 2069–2077.
6. Strieter, R. M., Kunkel, S. L., Arenberg, D. A., Burdick, M. D., and Polverini, P. J. (1995) Interferon gamma-inducible protein 10 (IP-10), a member of the C-X-C chemokine family, is an inhibitor of angiogenesis. *Biochem. Biophys. Res. Commun.* **210,** 51–57.
7. Koch, A. E., Polverini, P. J., Kunkel, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M., Elner, S. G., and Strieter, R. M. (1992) Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* **258,** 1798–1801.
8. Graham, G. J., Wright, E. G., Hewick, R., Wolpe, S. D., Wilkie, N. M., Donaldson, D., Lorimore, S., and Pragnell, I. B. (1990) Identification and characterization of an inhibitor of haemopoietic stem cell proliferation. *Nature* **344,** 442–444.
9. Nagasawa, T., Hirota, S., Tachibana, K., Takaura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996) Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* **382,** 635–638.

10. Sarris, A. H., Broxmeyer, H. E., Wirthmueller, U., Karasavvas, N., Cooper, S., Lu, L., Krueger, J., and Ravetch, J. V. (1993) Human interferon-inducible protein 10: expression and purification of recombinant protein demonstrate inhibition of early human hematopoietic progenitors. *J. Exp. Med.* **178,** 1127–1132.
11. Van Snick, J., Houssiau, F., Proost, P., Van Damme, J., and Renauld, J. C. (1996) I-309/T cell activation gene 3 chemokine protects murine T cell lymphomas against dexamethasone induced apoptosis. *J. Immunol.* **157,** 2570–2576.
12. Cook, D. N., Beck, M. A., Coffman, T. M., Kirby, S. L., Sheridan, J. F., Pragnell, I. B., and Smithies, O. (1995) Requirement of MIP-1α for an inflammatory response to viral infection. *Science* **269,** 1583–1585.
13. Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P. (1995) Identification of RANTES, MIP-1α, and MIP-1β as the major HIV-suppressive factors produced by CD8+ T cells. *Science* **270,** 1811–1815.
14. Peled, A., Gonzalo, J. A., Lloyd, C., and Gutierrez-Ramos, J. C. (1998) The chemotactic cytokine eotaxin acts as a granulocyte-macrophage colony-stimulating factor during lung inflammation. *Blood* **91,** 1900–1916.
15. Ponath, P. D. (1998) Chemokine receptor antagonists: novel therapeutics for inflammation and AIDS. *Exp. Opin. Invest. Drugs* **7,** 1–18.
16. Murphy, P. M. (1994) The molecular biology of leukocyte chemoattractant receptors. *Annu. Rev. Immunol.* **12,** 593–633.
17. Arai, H., Tsou, C.-L., and Charo, I. F. (1997) Chemotaxis in a lymphocyte cell line transfected with C-C chemokine receptor 2B: Evidence that directed migration is mediated by bg dimers released by activation of G_{ai}-coupled receptors. *Proc. Natl. Acad. Sci. USA* **94,** 14,495–14,499.
18. Neptune, E. R. and Bourne, H. R. (1997) Receptors induce chemotaxis by releaseing the bg subunit of Gi, not by activating Gq or Gs. *Proc. Natl. Acad. Sci. USA* **94,** 14,489–14,494.
19. Nieto, M., Frade, J. M. R., Sancho, D., Mellado, M., Martinez-A, C., and Sanchez-Madrid, F. (1997) Polarization of chemokine receptors to the leading edge during lymphocyte chemotaxis. *J. Exp. Med.* **186,** 153–158.
20. Ponath, P. D., Qin, S., Ringler, D. J., Clark-Lewis, I., Wang, J., Kassam, N., Smith, H., Shi, X., Gonzalo, J. A., Newman, W., Gutierrez-Ramos, J. C., and Mackay, C. R. (1996) Cloning of the human eosinophil chemoattractant, eotaxin. Expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils. *J. Clin. Invest.* **97(3),** 604–612.
21. Ponath, P. D., Qin, S., Post, T. W., Wang, J., Wu, L., Gerard, N. P., Newman, W., Gerard, C., and Mackay, C. R. (1996) Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. *J. Exp. Med.* **183(6),** 2437–2448.
22. Heath, H., Qin, S., Rao, P., Wu, L., LaRosa, G., Kassam, N., Ponath, P. D., and Mackay, C. R. (1997) Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. *J. Clin. Invest.* **99,** 178–184.

13

Endothelial Cell Chemotaxis Assays

Darcey Black

1. Introduction

Endothelial cell migration and proliferation play an important role in the growth and development of new blood vessels, in the process known as neovascularization or angiogenesis. In the normal developing embryo, new blood vessels are formed by the directed movement of endothelial cells from adjacent capillaries ***(1)***. A similar process occurs in many other normal physiological responses. These include wound healing, ovarian follicle maturation, the inflammatory response, and in immune reactions. However, angiogenesis is also a feature of a number of pathological states, and these include diabetes, atherosclerosis, arthritis, psoriasis, and cancer. In several instances the angiogenic process appears poorly controlled and results in the formation of leaky blood vessels. In other pathological states, such as cancer, the tumor itself appears to tightly regulate new vessel formation which results in its maintenance, and often continued growth.

The tight control of angiogenesis is postulated to result from a balance between the angiogenic, peptide-growth factors, such as vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF) and angiostatic factors such as TGFβ. Recently, however, members of the CXC chemokine family have also been shown to have both angiogenic and angiostatic properties. IL-8 is a potent angiogenic factor, whereas PF4 is angiostatic ***(2–4)***. This difference in activity appears to be explained by the absence of a specific amino acid sequence Glu-Leu-Arg (the ELR motif) in PF4. Other CXC chemokines such as IP-10 and mutants of IL-8, both lacking the ELR motif, are also angiostatic ***(5)***. It is not yet known through which receptor all of these chemokines are acting. However, ECs have been shown to

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express receptors for IL-8 and other chemokines such as SDF-1α ***(6,7)***. The presence of two CC chemokine receptors, CCR-2B and CCR-4 on ECs associated with the neovasculature has also been reported. These receptors bind several CC chemokines including MIP-1α, RANTES, and MCP-1. It has also been demonstrated that RANTES and MCP-1 are capable of stimulating chemotaxis of human umbilical vein endothelial cells (HUVECs) (Black, Power and Meyer, unpublished results). Therefore, it would appear that chemokines are capable of stimulating endothelial cell migration, and may be involved in a number of normal and pathological process.

There are two assays that have been used to measure endothelial cell chemotaxis in response to different stimuli. In the first, the phagokinetic assay, endothelial cells are coated onto the coverslips that are evenly coated with fine particles of coloidal gold or small plastic spheres ***(8)***. When the cells move in response to the chemotactic agent clear tracks are left behind them which reflect the movement of the cells. It is possible to carry out quantitative analysis by measuring the dimensions of the phagokinetic tracks using image analysis. However, the most widely used and perhaps best known assay for measuring cell migration is the modified Boyden chamber assay ***(9,10)***. The assay has been widely used for studies in neutrophil and leukocyte migration, but has more recently been used for similar studies with endothelial cells, epithelial cells, and smooth muscle cells. The Boyden chamber consists of two chambers separated by a porous filter. Migrating cells are able to pass through these pores. The chemoattractant is placed in one chamber and the control substance is placed in the other, thus establishing a chemoattractant gradient. The movement of cells from the control side to that containing the chemoattractant results from either chemokinesis or chemotaxis. Therefore, it is important to include the proper controls to distinguish between these processes. Compared to the phagokinetic method, the Boyden chamber is relatively easy to use, requires no expensive equipment and is able to provide quantitative data. It is primarily suited to use with rapidly moving leukocytes, but has been successfully adapted for use with the much slower moving ECs. Nevertheless the breakdown of the chemotactic gradient is likely to occur over time, and so it makes it difficult to run these studies for longer than 8–12 h.

Quantitative assessment of the cell migration is possible by a number of methods. The most widely used and simplest is to count the cells which have migrated through the filter in several microscopic fields (usually at least 3 at ×400 magnification). Alternatively, various spectroscopic methods have been developed, in which the number of migrated cells are measured by quantifying a stain extracted from the nuclei of the migrated cells ***(11)***. More sophisticated (and expensive) computer-assisted imaging systems can be used. However,

care must be taken to ensure that whatever imaging methodology is used for this, that it must be capable of distinguishing between the cells and the pores through which they have migrated.

Finally, several peptide growth factors are well established chemoattractant for endothelial cells, and can be used as positive controls in migration assays. These include bFGF and VEGF in the concentration range 10–100 ng/mL.

2. Materials

2.1. Apparatus

Multi-well modified Boyden chambers can be obtained from Neuro Probe Inc. (Gaithersburg, MD). Model AP48 has been widely used for endothelial cell chemotaxis assays. The apparatus consists of top and bottom acrylic plates, a silicon gasket and assembly screws. The bottom plate has 48 wells, each with 25 μL final volume. These correspond to holes on the top plate, and form the upper wells when the chamber is assembled. The filter (polycarbonate, 25 × 80 mm) is placed between the top and bottom plates, and a gasket is placed over the filter to create the seal. The apparatus can be purchased with a selection of accessories, such as curved forceps, filter clamps, and wipers. These are required to process the filters after use. Filters can also be obtained from Nucleopore Inc. (Pleasanton, CA) and Costar (Cambridge, MA).

2.2. Cells and Cell Culture

Primary cultures of venous and arterial endothelial cells can be prepared from fresh tissue by well-established methods. However, this is a time consuming and laborious process requiring a good source of tissue. Alternatively, good quality and well-characterized endothelial cells are now readily available from commercial sources, such as the American Type Culture Collection or Clonetics Inc. (Walkersville, MD). The latter currently can supply a range of ECs from different tissues, and from a variety of species.

Endothelial cells are maintained in a basal culture medium, such as DMEM or a proprietary medium supplied with the cells, which is supplemented with hydrocortisone (1μg/mL), epidermal growth factor (EGF; 100 μg/mL) bovine fibroblast growth factor (FGF; 1 ng/mL), antibiotics (gentamicin and amphotericin, at 50 μg/mL) and fetal calf serum (2–10%). Alternatively, bovine brain extract (3 μg/mL) can be used instead of EGF and FGF. The cells are cultured in either standard tissue culture flasks directly on plastic or on flasks coated with collagen. The cells are grown to confluence and subcultured and reseeded in a ratio of 1 : 3. For all experiments primary endothelial cells should be used between passages 3 and 12.

2.3. Solutions

Field's A: Methylene Blue, 0.8g, Azur I, 0.5g, Na_2HPO_4, 5.0g, KH_2PO_4, 6.25g, Distilled water, 500 mL. Field's B: Eosin, 1 g, Na_2HPO_4, 5.0 g, KH_2PO_4, 6.25 g, Distilled water, 500 mL.

3. Methods

The method used to perform the chemotaxis assay on endothelial cells is essentially that previously described for neutrophils and leukocytes, with one major difference. With neutrophils the cells are placed in suspension in the upper well and the chemotactic agent is placed in the lower well. However, with endothelial cells, the cells are placed in suspension in the lower well, and the filter and upper well put in place. The apparatus is then inverted, and left in an incubator for 2–4 h to allow the cells to adhere to the filter. After this period of incubation, the chambers are inverted again, back to their original orientation. The medium in the upper well is then replaced with fresh medium containing the chemotaxin, and experiment is allowed to proceed.

Two types of filters are available: polycarbonate and cellulose nitrate. Polycarbonate filters are used for endothelial chemotaxis assays. These filters are sided, with a matt and a shiny surface. The cells are allowed to adhere to the shiny surface prior to migration, and then stimulated to migrate to the matt surface.

3.1. Chemotaxis Assay

1. Put 4×10^5 cells are 28 μL of medium containing 1% BSA in each well in the lower compartment of the 48-well Boyden Chemotaxis chamber.
2. Overlay the wells with a 5-μm polycarbonate filter coated with 0.2 % gelatin.
3. Invert the chemotaxis chambers and incubate for 2 h at 37°C to allow the cells to attach to the membrane.
4. Turn the chambers back to their original orientation. Add 50 μL of medium containing the chemotactic agent to the top well.
5. Incubate for 4–6 h at 37°C.
6. At the end of the incubation period, carefully remove the filters from the apparatus and hold the filter at either end with forceps.
7. Hold the matte side of the filter uppermost. Place the shiny (lower) side of the filter in phosphate-buffered saline (PBS). Do not immerse the filter. Gently pull the wet surface of the filter over a rubber wiper to remove the cells that have not migrated through the pores.
8. Immerse the filter in 100 % (v/v) methanol for 1 min to fix the cells.
9. Place the filter on a microscope slide for support, and allow to dry at room temperature.

3.2. Staining and Quantitation

A number of methods are available. The author routinely used a computer assisted image analysis system. The cells which had migrated were visualized by staining the nuclei with Field's B solution. The reddish blue spots are then digitized and quantified.

3.2.1. For Image Analysis

1. Place the filter in a Petri dish.
2. Cover the membrane with 5 mL of Field's B solution.
3. Add 5 mL of Field's A solution and mix well.
4. After about 1 min remove the filter and wash thoroughly in distilled water.
5. Leave the filter to dry at room temperature for at least 1 h.
6. Place immersion oil on the membrane view under high power (×400).
7. Using an appropriate image analysis program, quantitate the number of cell nuclei which appear as red spots.
8. The results can be expressed in terms of the numbers of cells migrating in a given field, area of the digitized image of the migrated cells (pixels) or the chemotactic index. The chemotactic index is estimated as number of cells migrating in response to medium containing the chemotactic factor/number of cells migrating to medium alone.

3.2.2. For Manual Counting

1. Cells are stained with Diff-Quick (Baxter Health Care, McGaw Park, IL) for 1 min.
2. Cells that have migrated are counted in at least 10 high powered fields (×400) using a standard microscope, and the mean value calculated.

3.2.3. For Spectrophometric Analysis

1. Remove the filters from the glass slides and place in distilled water in a Petri dish for up to 2 min.
2. Place the filters in the well of a 96-well plate and add 300μL of 0.1*M* HCl and mix well.
3. Transfer 250 μL of the solution to a second 96-well plate and measure the absorbance at 600 nm.
4. Preliminary studies should be carried out to establish the correlation between the number of endothelial cells and the absorbance at 600 nm.

4. Notes

The Boyden chamber method adapted for endothelial cells has proven to be simple, relatively robust, and reproducible in a number of laboratories. There are several variations on the basic method which have been reported in the literature. However, the precise method ultimately adopted will depend on

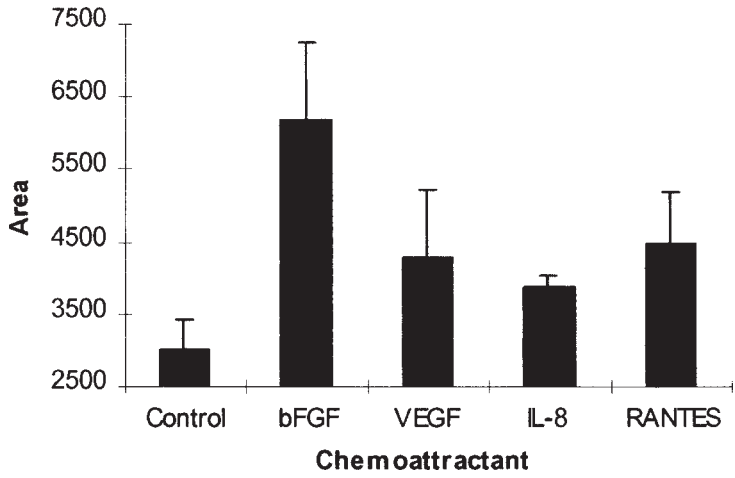

Fig. 1. The chemotaxis of human umbilical vein endothelial cells in response to bFGF (50 ng/mL), VEGF (50ng/mL), and the chemokines IL-8 (50 n*M*) and RANTES (100 n*M*). The assay was carried out using filters with a pore size of 8 μ*M*, and the migration assay was carried out for 6 h. The results are given as area of the digitized image of the migrated cells (pixels), using a Sony video camera and a Kontron Vidas image analysis system.

the outcome of preliminary studies, and the detection method of choice. Typical results obtained by the author are illustrated in **Fig. 1**.

However, there are a number of points to consider when carrying out endothelial cell chemotaxis assays.

1. Each data point should be carried out in at least triplicate. This allows for a maximum of 16 data points per chemotaxis chamber (48 wells in total). The design of the apparatus ensures that there is little cross-contamination between wells. In addition, the wells are arranged to ensure that it is easy to orientate the filter and identify individual wells.
2. The optimum pore size for endothelial cell migration should be determined. A number of sizes have been reported in the literature ranging from 5–12 μm in diameter. Filters with pore sizes of 5, 8, 10, and 12 μm are currently available from manufacturers. It is advisable to check the appropriate size, which may be dependent on the tissue source of the endothelial cell and the method of preparation of the cells. The author has routinely used 8 μm pore size.
3. It is also necessary to determine the optimal time over which to measure the migration of the cells, since it is important to establish a sufficient window to allow the accurate measurement of the migration. Since endothelial cells are relatively slow moving compared to neutrophils or leukocytes, longer incubation times are required to allow sufficient cells to migrate to allow accurate quantitation. Reports in the literature vary from 2–6 h. Therefore, it is important

to determine the optimal migration time for each set of conditions and endothelial cell type.

4. It is important to establish that the apparent migration in response to a putative chemoattractant is due to chemotaxis, chemokinesis or a combination of both processes. This is established by means of a simple "checkerboard" assay. In this assay, the same concentration of the putative migration factor is added to both the upper and lower compartment of the chamber, over a set concentration range. The cells are added to one side, and those migrating to the other side are measured. The number of cells migrating under these conditions represent those which have migrated due to the process of chemokinesis, i.e., their migration is due to random and not directional movement, and does not depend on a concentration gradient.

References

1. Folkman, J. (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature Med.* **1,** 27–31.
2. Koch, A. E., Polverini, P. J., Kunkel, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M., Elner, S. G., and Strieter, R. M. (1992) Interleukin 8 as a macrophage-derived mediator of angiogenesis. *Science* **258,** 1798–1801.
3. Smith, D. R., Poleverini, P. J., Kunkel, S. L., Orringer, M. B., Whyte, R. I., Burdick, M. D., Wilke, C. A., and Strieter, R. M. (1994) Inhibition of IL-8 attenuates angiogenesis in bronchogeneic carcinoma. *J. Exp. Med.* **179,** 1409–1415.
4. Maione, T. E., Gray, G. S., Petro, J., Hunt, A. J., Donner, A. L., Bauer, S. I., Carson, H. F., and Sharpe, R. J. (1990) Inhibition of angiogenesis by recombinant human PF4 and related peptides. *Science* **247,** 77–79.
5. Strieter, R. M., Polverini, P. J., Kunkel, S. L., Arenberg, D. A., Burdick, M. D., Kasper, J., et al. (1995) The functional role of the ELR motif in CXC chemokine-mediated angiogenesis *J. Biol. Chem.* **270,** 27,438–27,357.
6. Schonbeck, U., Brandt, E., Petersen, F., Flad, H.-D., and Loppnow, H. (1995). Il-8 specifically binds to endothelial but not smooth muscle cells. *J. Immunol.* **195,** 2375–2383.
7. Gupta, S. K., Lysko, P. G., Pillarisetti, K., Ohlstein, E., and Stadel, J. M. (1998) Chemokine receptors in human endothelial cells. *J. Biol. Chem.* **273,** 4282–4287.
8. Zetter, B. R. (1987) An assay of capillary endothelial cell migration, in *Methods in Enzymology*, vol. 147 (Barnes, D. and Sirbasku, D.A., eds.), Academic, New York, pp. 135–144.
9. Falk, W., Goodwin, R. H., and Leonard, E. J. (1980) A 48-well micro chemotaxis assay for rapid and accurate measurement of leukocyte migration. *J. Immunol. Methods* **33,** 239–247.
10. Richards, K. L. and McCullough, J. (1984) A modified microchamber method for chemotaxis and chemokinesis. *Immunolog. Commun.* **13,** 46–62.
11. Grotendorst, G. R. (1987) A spectrophotometric assay for the quantitation of cell migration in the Boyden chamber chemotaxis assay, in *Methods in Enzymology*, vol. 147 (Barnes, D. and Sirbasku, D. A., eds.), Academic, New York, pp. 144–152.

14

Radiolabeled Chemokine Binding Assays

Bruce L. Daugherty, Salvatore J. Siciliano, and Martin S. Springer

1. Introduction

Knowledge of a receptor's pharmacology provides information which is often essential to unraveling the biological function of that receptor. The chemokine system represents a striking example as most receptors in the family bind multiple chemokines, often with complex overlapping selectivities. Two basic methodologies are used to characterize the binding properties of a receptor. In saturation protocols, the binding of a given ligand, usually labeled to allow detection, is determined over a range of concentrations appropriate for that ligand. Competition experiments measure the ability of varying concentrations of an unlabeled ligand to inhibit the binding of a fixed concentration of a labeled ligand. Although saturation protocols have the advantage of theoretical simplicity, they have three significant disadvantages:

1. The signal-to-noise ratio varies with concentration of the ligand, usually decreasing dramatically with increasing concentration;
2. A labeled analog must be generated for each ligand to be examined;
3. The binding characteristics determined are those of the labeled analogs, which can differ substantially from the natural ligands.

Competition protocols eliminate these disadvantages and careful attention to experimental design and modern computer analysis minimize difficulties resulting from their greater theoretical complexity.

Labeled chemokines have been generated by a variety of procedures including fusion with alkaline phosphatase ***(1)***, derivatization with fluorescent probes ***(2)***, and radiolabeling ***(3,4)***. While homogeneous fluorescent based assays (fluorescence polarization, fluorescence energy transfer, etc.) will become

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more prevalent in the future, most current assays use iodinated chemokines due to their commercial availability (New England Nuclear Boston, MA; Amersham, Arlington, Heights, IL) and ease of preparation ***(3,4)***. The radiolabeled assays can be further subdivided into homogenous mix and read assays (Scintillation Proximity), and those that require a separation of free from bound ligand. The former provide for experimental simplicity, whereas the latter, at least in our hands, have better signal-to-noise ratios (S/N) and substantially lower costs. Although the rest of this chapter will focus on competition protocols employing a separation procedure and iodinated chemokines, most of the experimental design is applicable to any of the methodological variants.

In designing a competition based assay, theoretically, it's best to keep the concentrations of both receptor and labeled ligand as low as possible, and well below K_d. This is not always possible as the level of nonspecific background and the need to maintain a S/N ratio sufficient to generate data of good precision often dictate the conditions used. Thus, it is important to minimize background. With appropriate choice incubation and wash buffers, and filters (or beads for SPA) the nonspecific backgrounds can be kept to 1–2% of the total input labeled ligand used for most chemokines. To obtain a S/N of 5–10 about 10% of the labeled chemokine should be bound requiring a receptor concentration of ~0.2 K_d (*see* **Note 1**). Although the level of receptor used may be dictated by nonspecific binding, the concentration of radiolabeled ligand should be kept as low as possible while binding no more than 20% of the ligand and maintaining an adequate signal. The slope of the binding isotherm (on linear scale) is maximal at zero ligand concentration and decreases with increasing concentration. In contrast, nonspecific background usually increases in a linear fashion with concentration. Thus, the lower the labeled ligand concentration the higher the S/N. Once the general parameters of the assay have been established the kinetics of binding are measured to establish the incubation time required to attain equilibrium.

The importance of proper experimental design is illustrated in **Fig. 1**, which shows two competition experiments using MIP-1α and human CCR1 ***(5,6)***. The two protocols employ an rat basophilic leukemia (RBL) cell line stably expressing CCR1 at 2×10^5 receptors/cell as the source of the receptor, and differ only in the number of cells used. To generate the curve defined by the open circles, 10^5 cells/point were used, or a receptor concentration of ~0.15 n*M*. The experiment gave an IC_{50} of 0.2 n*M*, a value very close to the true Ki. The second experiment (solid circles) used 10^6 cells/point, or a receptor concentration of 1.5 n*M*, and gave an IC_{50} of 3–4 n*M*. The steep slope of the second curve indicates that one or more of the parameters utilized were not appropriate. Whereas an analysis program such as LIGAND ***(7)*** can, theoretically,

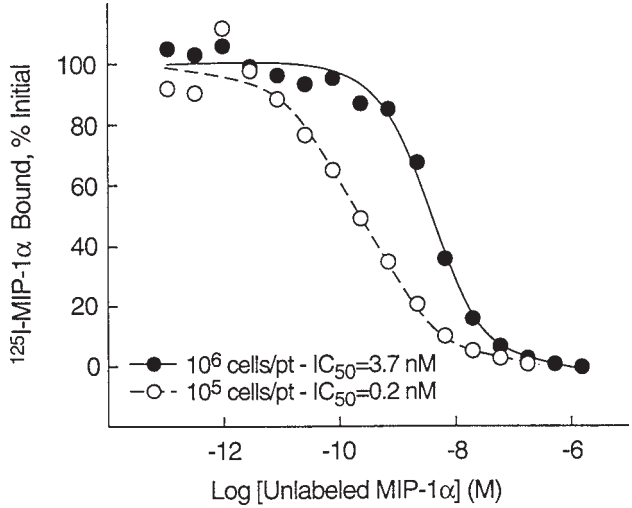

Fig. 1. Binding assay using increasing concentrations of unlabeled MIP-1α to compete against 25,000 dpm (30 pM) of ^{125}I-MIP-1α as described. CCR1 was recombinantly expressed in RBL-2H3 cells. The experiment shows the results obtained using either 10^5 cells/point (○), or 10^6 cells/point (●). See text for details.

compensate for the too high receptor concentration employed, in practice the results tend to be rather inaccurate since the large extrapolations require more precise knowledge of reagent concentrations than one usually has. Thus, accurate determinations of K_i are best made by designing the experiments properly, and not relying on computer compensation.

Typically there is some uncertainty in the concentration of the labeled reagent. Although one can accurately determine the amount of radioactivity employed, and usually the specific activity is known with precision, a significant fraction of the labeled reagent may be inactive. These reagents are made by chemical modification, procedures which lead to heterogenous products. In our hands most of the iodinated ligands contain no more than 50% active material. If the concentration of ligand used is below K_d, and only a small fraction is bound in the experiment, this uncertainly is of no consequence. However, if the experimental conditions are such that a large fraction of the ligand is bound, errors in determining K_i may well result. In fact, some of the difference between the two curves shown in **Fig. 1** resulted from binding all of the available ligand in the second experiment (solid circles). It is usually best to determine what fraction of the labeled ligand is capable of binding to the receptor. This can readily be accomplished by adding increasing amounts of receptor to a fixed concentration of labeled ligand until maximal binding is reached.

2. Materials

1. Receptor.
   a. Typically experiments are done with whole cells in which the receptor is recombinantly expressed, or with membranes prepared from those cells. However, binding experiments can also be carried out with primary cells, although greater cell numbers are usually required due to lower expression levels.
   b. In the experiments described here chemokine receptor CCR1 has been recombinately expressed in RBL-2H3 cells ***(8)***.
2. Chemokines (*see* **Note 1**).
   a. Unlabeled chemokines are available from a number of commercial sources, and depending on the source have been generated recombinantly or by peptide synthesis.
   b. Iodinated chemokines with a typical specific activity of 2200 Ci/mmole (1 iodine/molecule) are available from either Amersham or New England Nuclear. A variety of procedures for iodinating have also been described ***(3,4)***.
3. Incubation buffer is 50 m*M* HEPES, pH 7.2, containing 0.5% BSA, 5 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 10 μg/mL each of chymostatin, leupeptin, aprotinin, and 0.1 m*M* PMSF. All inhibitors are maintained as 1000X stocks and added to the buffer just prior to use. Chymostatin stock is in DMSO, aprotinin and leupeptin stocks are in water, while PMSF is in methanol.
4. Hanks balanced salt solution (HBSS), buffered with 25 m*M* HEPES at pH 7.2, and containing 0.5% BSA.
5. 0.33% polyethyleneamine (PEI).
6. Wash buffer is 25 m*M* HEPES, pH 7.2, containing 0.5*M* NaCl.
7. Incubations are carried out either in u-bottom microtiter plates, or in individual 12 × 75 mm test tubes. Typically these are made of polypropylene, although polystyrene vessels may also be used.
8. Shaker.
9. Whatmann GF/C filters or Packard Unifilter 96 GF/C 96-well filter plates.
10. Filtration device.
    a. When doing small numbers of assays in individual tubes, or when the assays require very high cell numbers, which would clog automatic harvestors ($>5 \times 10^5$/point), filtration is carried out using a Hoeffer manifold and 25-mm circular GF/C filters.
    b. When doing large numbers of assays in microtiter plates, filtration is carried out using a Packard Filtermate 196 Harvestor.
11. Vacuum source suitable for filtration; while a small pump is preferable, house vacuum will often suffice.
12. Microscint 0 from Packard Instruments (Downers Grove, IL).
13. Appropriate scintillation or γ-counter.

3. Methods

1. Cells are harvested, washed and stored on ice in HEPES buffered HBSS. Cells are stored at a concentration such that 20 μL contains the number appro-

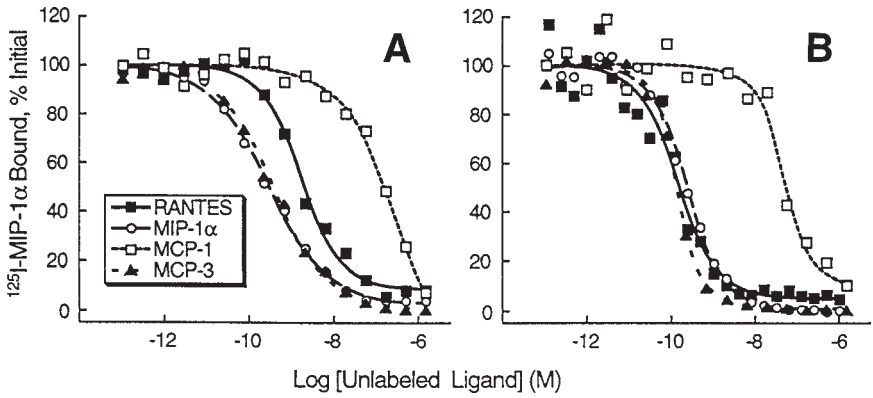

Fig. 2. Binding experiment using a variety of chemokines to compete against ^{125}I- MIP-1 on CCR1. **(A)** intact cells as the source of the receptor; **(B)** membranes as the source of the receptor. Experiment was performed as described.

priate for an assay. In the experiment shown in **Fig. 2**, they were stored at 5×10^6 cells/mL.

2. 200 μL of incubation buffer are added to wells in a 96-well plate.
3. 20 μL of ^{125}I-chemokine at 2200 Ci/mmole (MIP-1α in this experiment) are added to each well. The labeled chemokine has been diluted into incubation buffer so that 20 μL contains 20,000–30,000 DPM.
4. 5 μL of the unlabeled chemokine or other competitor are added. Dilutions have been previously made so that 5 μL delivers the desired concentration. If the competitor is a chemokine or other water soluble molecule the dilutions are made into incubation buffer. If the competitor is not water soluble, the dilutions are made into DMSO. In our hands all chemokine binding assays will tolerate >5% DMSO.
5. The plate is shaken to assure appropriate mixing of all components, and the binding reaction is started by adding 20 μL of cells.
6. The plates are placed on a shaker and incubated at room temperature for 90 min.
7. Filter plates are briefly soaked by dipping in 0.33% PEI, and just prior to use shaken to remove excess PEI and blotted.
8. The cells are harvested with the Packard Filtermate and washed as follows. After placing the plates on the harvester, the samples are aspirated, the wells then refilled with wash buffer, and aspirated again. The wash is repeated, and aspiration continued for about 20 s at which time the filter plate is released, the vacuum terminated, and the filter plate removed from the harvester.
9. The plate is placed in an incubator and dried at 45°C for 20 min. After removing the plate the bottom is sealed, 30 μL of Microscint O added to each well, the top sealed, the plate counted in Packard Topcount.

Results of a typical experiment using this protocol are shown in **Fig. 2**.

4. Notes

1. When using ligand concentrations well below K_d the receptor levels necessary to bind a given fraction of that ligand can be approximated from the following relationship: fraction of input ligand bound = [receptor]/([receptor] + K_d) where [receptor] is the free concentration of receptor in the same units as K_d.
2. If whole cells are utilized, the experimental conditions should preclude receptor internalization if true equilibrium is to be established.
3. Care should be exercised as to the quality of reagents. We always obtain a mass spectral analysis of each batch of chemokine used, as well as an N-terminal sequence.

References

1. Hieshima, K., Imai, T., Opdenakker, G., Van Damme, J., Kusuda, J., Tei, H., Sakaki, Y., Takatsuki, K., Miura, R., Yoshie, O., and Nomiyama, H. (1997) *J. Biol. Chem.* **272,** 5846–5853.
2. Inglese, J., Samama, P., Patel, S., Burbaum, J., Stroke, I. L., and Appell, K. C. (1998) *Biochem.* **37,** 2372–2377.
3. Bennett, G. L. and Horuk, R., in *Methods in Enzymology,* vol. 288 (Horuk, R., ed.), Academic, New York, NY.
4. Van Riper, G., Siciliano, S., Fischer, P. A., Meurer, R., Springer, M. S., and Rosen, H. (1993) *J. Exp. Med.* **177,** 851–856.
5. Neote, K., DiGregorio, D., Mak, J. Y., Horuk, R., and Schall, T. (1993) *Cell* **72,** 415–425.
6. Gao, J.-Y., Kuhns, D. B., Tiffany, H. L., McDermott, D., Li, X., Francke, U., and Murphy, P. M. (1993) *J. Exp. Med.* **177,** 1421–1427.
7. Munson, P. J. and Rodbard, D. (1980) *Anal. Biochem.* **107,** 220–239.
8. Siraganian, R. P., McGivney, A., Barsumian, E. L., Crews, F. T., Hirata, F., and Axelrod, J. (1982) *Fed. Proc.* **41,** 30–34.

15

Scintillation Proximity Binding Assay

Sami Alouani

1. Introduction

Receptor-ligand interactions have been extensively used to establish the specificity and binding profiles for chemokine receptors and their ligands. Furthermore, receptor-ligand binding assays are used extensively for drug screening. The Scintillation Proximity Assay (SPA) is now a well established screening technology and a considerable number of SPA based receptor screening assays have been run by pharmaceutical companies. This homogeneous radioisotopic technique offers considerable advantages in terms of convenience, safety, and cost. The method utilizes scintillant-containing microspheres that are chemically treated to enable the coupling of molecules (e.g., antibodies, receptors, and enzymes) to their surface. Assays are carried out in aqueous buffers using radioisotopes that emit low energy radiation which is easily dissipated in an aqueous environment.

The beta particles emitted by ^{3}H and the Auger electrons released from ^{125}I by isotopic decay have average energies of 6 and 35 keV, respectively, and thus have short pathlengths in water. This property makes them ideal for use with the SPA technology. If a molecule labeled with ^{3}H or ^{125}I is bound to the surface of the bead, directly or via interaction with a previously coupled molecule, it is in close enough proximity for the emitted radiation to activate the scintillant contained within the bead and produce a light signal. The amount of light produced, which is proportional to the amount of labeled molecules bound to the beads, can be measured by a scintillation counter. The energy of the beta particles released from molecules not attached to the surface of the beads is absorbed by the aqueous solvent before it reaches the beads and no light is produced (**Fig. 1**).

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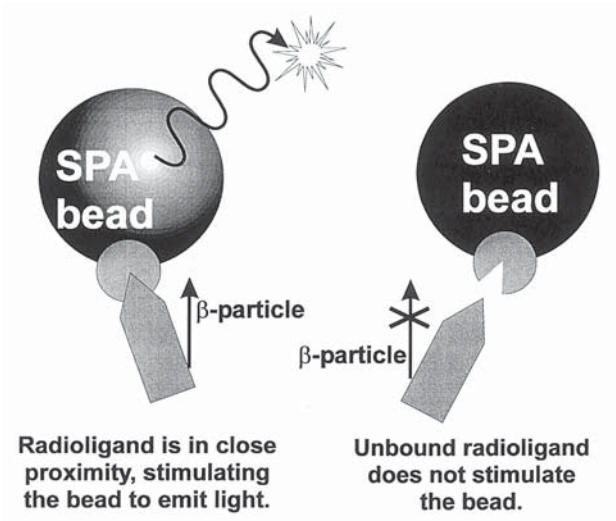

Fig. 1. Diagrammatic representation of Scintillation Proximity Assay.

SPA has been developed as a receptor binding assay using molecules prepared from a number of different sources ranging from crude tissue preparations to soluble purified proteins. The method, which is applicable to both ^{3}H and ^{125}I ligands, provides a number of factors and must be carefully optimized in the assay design and development.

SPA is a homogeneous assay technique and has the big advantage of not requiring washing steps. The lack of separation steps is also a considerable advantage when assaying receptors of weak affinity where filtration or washing steps may shift the apparent equilibrium of the binding event under study. This means also that SPA assay is fast, simple, and easy to automate. Thus, a large number of samples can be measured due to the stability and reproducibility of the assay, making it an ideal assay for robotic systems.

In traditional methods, the assay is often terminated prior to the attachment of equilibrium, and the samples counted. In SPA, however, it is important that the assay is allowed to reach equilibrium before counting is performed. Since this requires a more prolonged incubation period, it is essential to ensure that the different components used are stable.

Two other aspects of the assay should also be considered:

1. The measurement of light in colored samples: this can be overcome by color quench correction programs applied by the appropriate scintillation counter.
2. Because SPA is a solid phase technology, it is important to optimize the amount of receptor with respect to the immobilizing surface. The surface area of the

beads is finite and the signal attainable therefore depends on the number of receptors bound to the beads, the affinity of the ligand for the receptor, and the specific activity of the ligand used.

The establishment of an SPA receptor assay requires similar steps to those required by traditional methods. The process can be separated into the following steps:

1. Selection of ligand and receptor source.
2. Selection of assay buffer, bead type, and assay format.
3. Optimization of membrane : bead ratio.
4. Optimization of ligand concentration.
5. Determination of the counting window.
6. Assay validation.

In the following sections, both membrane preparation and SPA assay optimization for chemokine-chemokine receptor interactions will be described.

There are two types of SPA binding assays which can be carried out:

1. In order to determine the K_D, a saturation curve can be obtained by varying the labeled ligand concentration. This requires measuring both total and nonspecific binding (**Fig. 2A**).
2. In order to determine the IC_{50}, competition can be obtained by varying the nonlabeled ligand concentration to obtain a curve of competition equilibrium (**Fig. 2B**).

2. Materials

2.1. Membrane Preparation

Prepare all buffers from analytical grade reagents and dissolve in water. The solutions should be stored at 4°C and are stable for 1 mo.

1. Cell culture (*see* **Note 1**).
2. Homogenization buffer, pH 7.4. Concentrations given below indicate the final concentrations. The concentrations of stock solutions that can be used are indicated. 50 m*M* HEPES (stock solution 1 *M*), 1 m*M* EDTA 0.5 *M* (stock solution pH 8.0), 10 m*M* $MgCl_2$ (stock solution 1 *M*), Protease inhibitor cocktail tablets (*see* **Note 2**), 2 μ*M* Pepstatin A (stock solution 1 m*M* in 100% DMSO), 1 m*M* phenylmethylsulfonyl fluoride (PMSF) (stock solution 50 m*M* in 100% alcohol) (*see* **Note 2**).
3. Resuspension buffer: Same as homogenization buffer but without Pepstatin A and PMSF.
4. Equipment: Ultra Turax homogenizer or equivalent; Centrifuges to enable 500*g* and 48,000*g*; 50-mL tubes such as Falcon (Los Angeles, CA); 50-mL polypropylene tubes for high-speed centrifugation.

2.2. SPA Binding Assay

All buffers and solutions should be prepared from analytical grade reagents and dissolved in deionized water. The buffers can be stored at 4°C and are stable for 1 month. The beads are stable for several months at 4°C. Membranes are stable for several years at –80°C (*see* **Note 3**).

Concentrations given below indicate final concentration.

1. Binding buffer, pH 7.2: 50 m*M* HEPES (stock solution 1 *M*), 5 m*M* $MgCl_2$ (stock solution 1 *M*), 1 m*M* $CaCl_2$ (stock solution 1 *M*), 0.5% (w/v) BSA.
2. SPA Beads: 2.5 mg/mL WGA Beads (Amersham Pharmacia Biotech), resuspended at 50 mg/mL (*see* **Note 4**).
3. Membranes prepared as described above: 20 μg/mL.
4. Radiolabeled chemokine: 0.1 n*M* from a 23 n*M* stock solution at 2000 Ci/mMoles) (Amersham) (*see* **Note 5**).
5. Cold ligand for competition binding assay: Increasing concentration from a 1 mg/mL stock solution (*see* **Note 6**).
6. Cold ligand for saturation binding assay: 100–500 × the concentration of the radiolabeled chemokine.
7. Equipment: 96-well plate (PET flexible 1405–401 (Wallac, Turku, Finland) (*see* **Note 7**); shaker; Scintillation counter (Wallac) (*see* **Note 8**).

3. Methods

3.1. Membrane Preparation

1. Suspend the cells in cold homogenization buffer at 2×10^8 cells/mL.
2. Disrupt the cells using an Ultra Turax (maximum speed, 20,000*g*) for 30 s, waiting for 2 min on ice between each breakage. This should be done at least 3 times (*see* **Note 9**).
3. Centrifuge the cell suspension at 500*g* for 20 min at 4°C.
4. Aspirate the supernatant (the cell debris is in the pellet) and centrifuge for 30 min at 48,000*g* at 4°C.
5. Resuspend the resulting membrane pellet in the Resuspension buffer and pass through a 0.6 × 25 (mm) needle.
6. Aliquot the membrane suspension in Eppendorf tubes and freeze for storage at –80°C.
7. Protein determination is done on a thawed membrane aliquot just before using them for the SPA assay (*see* **Note 10**).

3.2. SPA Binding Assay

3.2.1. Saturation binding assay (see **Fig. 2A**)

3.2.1.1. Total Binding Assay

1. Prepare two- or threefold serial dilution of the radioligand to obtain final concentrations between 20 n*M* and 0 n*M*.

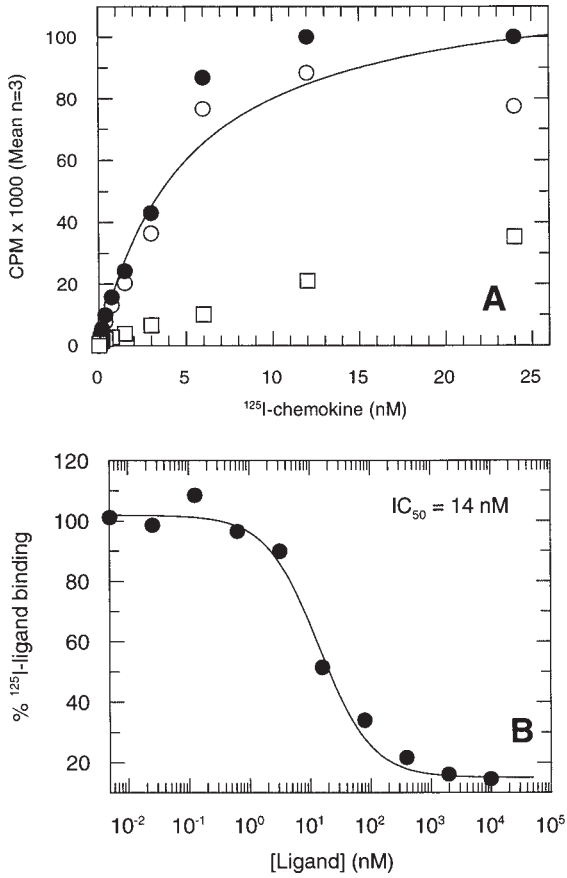

Fig. 2. **(A)** Saturation binding curve of ^{125}I-MIP-1 on recombinant CHO-CCR1 membrane using SPA technology. Binding assay were performed with increasing concentration of ^{125}I-MIP-1α as described. Total binding (●), non-specific binding (□) and specific binding (○). **(B)** Competition binding assay on CHO-CCR1 cell membrane using the SPA technology. Binding assay were performed as described.

2. Prepare wheat-germ agglutinin (WGA) beads in binding buffer at a concentration of 10 mg/mL.
3. Prepare membranes in binding buffer at a concentration of 80 μg/mL
4. Add to each well of a 96-well plate: 25 μL of the bead suspension; 25 μL of membrane preparation; 25 μL of binding buffer; 25 μL of diluted radioligand.
5. Shake the 2 plates at room temperature for at least 3 h (maximum overnight) and count each well for 1 min in the counter.
6. The assay should be done in triplicate (*see* **Note 11**).

3.2.1.2. Nonspecific Binding Assay

1. Prepare two- or threefold serial dilution of the radioligand to obtain final concentrations between 20 n*M* and 0 n*M*.
2. Prepare WGA beads in binding buffer at a concentration of 10 mg/mL.
3. Prepare membranes in binding buffer at a concentration of 80 μg/mL
4. Prepare 100-fold excess nonradiolabeled ligand in binding buffer.
5. Add to the 96-well plate (per well): 25 μL of beads; 25 μL of membrane preparation; 25 μL of nonradiolabeled ligand; 25 μL of diluted radioligand.
6. Shake the 2 plates at room temperature for at least 3h (maximum overnight) and count each well for 1 min in the counter.
7. The assay should be done in triplicate (*see* **Note 11**).

3.2.2. Competition Binding Assay (see **Fig. 2B)**

1. Prepare three- or fourfold serial dilution of the nonradiolabeled ligand in binding buffer.
2. Prepare 0.4 n*M* radiolabeled ligand in binding buffer.
3. Prepare 10 mg/mL WGA beads in binding buffer.
4. Prepare 80 μg/mL membrane in binding buffer.
5. Add to each well of a 96-well plate: 25 μL of beads; 25 μL membrane; 25 μL of the dilution of unlabeled ligand; 25 μL of the radioligand.
6. Seal the plate with an adhesive tape.
7. Shake at room temperature for a period of 3 h (minimum) to 16 h (maximum).
8. Count with a Wallac microbeta counter for 1 min/well.

The assay should be done in triplicate (*see* **Note 11**).

4. Notes

1. Membranes can be prepared from cells cultured in a fermentor at a concentration of approx 10^6 cells/mL or from 750-mL tissue culture flasks at 60–70% confluence.
2. The protease inhibitor cocktail tablets: "Complete" from Roche Molecular Biochemicals, Rotkreuz, Switzerland, Cat. No. 1 697 498. PMSF: Phenylmethylsulfonyl fluoride, Sigma Cat. No. P-7626.
3. Once a membrane aliquot is thawed, never refreeze.
4. WGA Beads: Wheat-Germ Agglutinin SPA beads. This lectin, as many lectins, has been shown to act by binding to sugar residues on the surface of the cells. Resuspend the beads thoroughly before use.
5. We use a final concentration of 0.1 n*M* of radiolabeled chemokine at 2000 Ci/mmole (7.4TBq/mmol). After reconstitution at 23 n*M* in water, it can be aliquoted and stored at –20°C. We dilute it 60-fold in binding buffer to obtain 0.4 n*M* and then add 25 μL/well.

   Chemokine radioligands are commercially available from Amersham or NEN. Amersham has established a Custom Labeling Service for ligands that are not

sold in their catalog. Alternatively, the ligands can be radiolabeled by standard procedures which are outside the scope of this chapter (Bolton-Hunter Reagent (SHPP), IODO-BEADS® Iodination Reagent, both kits from Pierce).

6. Chemokines are stored as lyophilized powders. Make up 1 mg/mL stock solution in deionized water. This can be stored frozen and diluted in binding buffer. The nonradiolabeled chemokines, most of which are available from commercial suppliers such as R&D Systems or PeproTech, or which can be prepared by methods described in other chapters in this volume (*see* Chapters 1,3–6,8) can be kept at –20°C after reconstitution at 1 mg/mL in water. Repeated freeze-thawing is not advisable, so the solutions should be aliquoted in small volumes suitable for single assays.
7. Solid 96-well plates can also be used (Wallac).
8. Two different beta counters can be used with the appropriate plates: Microbeta counter from Wallac or the Top Count from Packard (Downers Grove, IL).
9. To verify the state of the membrane preparation after sonication, mix 1 vol of the membrane with 1 vol of trypan blue and examine by microscopy. If after 3 sonications live cells are still found, continue to sonicate. 80–90% of the cells should take up the trypan blue before proceeding to the next step. Trypan blue stains dead cells.
10. The membrane concentration is measured using a colorimetric protein determination assay such as those supplied by Bio-Rad or Pierce.
11. All of the different products used in the assay should be freshly prepared.

References

1. Cook, N. D. (1992) SPA: a revolutionary new technique for drug screening. *Pharmaceutical Manufacturing International,* pp. 49–53.
2. Bosworth, N. and Towers, P. (1989) Scintillation proximity assay. *Nature* **341,** 167–168.
3. Takeushi, K. (1992) Scintillation proximity assay. *Lab. Practice* September issue.
4. Udenfriend, S., Gerber, L. and Nelson, N. (1987) Scintillation Proximity Assay, a sensitive and continuous isotopic method for monitoring ligand-receptor and antigen-antibody interactions. *Anal. Biochem.* **161,** 494–500.
5. Cook, N. D. (1996) Scintillation proximity assay—a versatile high throughput screening technology. *Drug Discovery Today* **1(7),** 287–294.
6. Nelson, N. (1987) A novel method for the detection of receptors and membrane proteins by scintillation proximity radioassay. *Anal. Biochem.* **165,** 287–293.
7. Banks, M. (1995) Soluble interleukin-5 receptor α chain binding assays: use for screening and analysis of interleukin-5 mutants. *Anal. Biochem.* **230,** 321–328.
8. Power, C. A. (1997) Cloning and characterisation of a specific receptor for the novel CC chemokine MIP-3α from lung dendritic cells. *J. Exp. Med.* **186(6),** 825–835.

16

Calcium Mobilization

Raphaële Buser and Amanda E. I. Proudfoot

1. Introduction

The intracellular ionic composition is closely related to cellular activity. The key roles in controlling cell metabolism are played by calcium, hydrogen, sodium, and potassium concentrations. It is generally recognized that cells spend a great amount of their energy on stringent regulation of the level of cytosolic free Ca^{2+}, and that Ca^{2+} plays a very important regulatory role in cellular functions.

The development of our knowledge of the intracellular behavior of ions lead to the following important findings. First, populations of cells are heterogeneous with respect to their average cytoplasmic ionic composition. It has been shown that cells in suspension show individual differences in pH_{in} or $pCa^{2+}{}_{in}$ values. Second, observing a greater number of cells individually for a long period revealed that the level of intracellular Ca^{2+} is not constant over time. In many cells, the Ca^{2+} concentration suddenly increases and then returns to the original value. Such spontaneous Ca^{2+} oscillations ("spikes") appear synchronously in most of the observed cells and repeat every several ten or hundred seconds. Third, the change in Ca^{2+} concentrations are local. It appears that the rise in Ca^{2+} concentration moves through the cytoplasm in a manner analogous to a wave.

It is recognized that there are two major pathways of entry of Ca^{2+} into the cells: rapid Ca^{2+}entry from the extracellular medium is regulated principally by macromolecules of the plasmic membrane, or Ca^{2+} may be released from the intracellular stores such as the Golgi, the endoplasmic and sarcoplasmic reticuli. In many cases, a Ca^{2+} signal is initiated by voltage- or receptor-gated opening of Ca^{2+} permeable plasmalemmal channels. Calcium channels can also

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be regulated by a phosphorylation mechanism that is activated by different protein kinases. These are generally activated by second intracellular messengers, themselves dependent on extracellular receptors.

A third mode of entry of Ca^{2+} into the cells is mediated by the coupling of the receptor to G proteins. It is believed that this is the pathway used by chemokines. The "On" reaction begins when the agonist induces a conformational change in the receptor which is transmitted to the G protein. The heterodimeric G protein dissociates into Gα and Gβγ subunits, both of which can activate different phospholipase C (PLC) isozymes. This enzyme is able to catalyze the hydrolysis of the lipid precursor phosphatidylinositol 4,5-bisphosphate to give both 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). The latter then binds to the IP_3 receptor which mobilizes both stored Ca^{2+} and promotes an influx of external Ca^{2+}.

1.1. Calcium Indicators

Earlier techniques for measuring cytosolic free Ca^{2+} ***(1–2)*** such as the luminescent photoprotein aequorin, the absorbance dye arsenazo III and Ca^{2+}-sensitive microelectrodes, all required microinjection or impalements, and were therefore applied mainly to giant cells. Later, photoproteins have been loaded with various reversible permeabilization procedures ***(3)***, but the largest expansion in the range of cell types in which Ca^{2+} signals can be quantified has come from the development of new fluorescent indicators that can be loaded using hydrolyzable esters. Currently four fluorescent indicators are used frequently: quin-2, fura-2, indo-1 and fluo-3.

Their structures share nearly identical binding sites, which are modeled on the Ca^{2+} selective chelator ethyleneglycol-bis-(β-aminoethylether)N,N'-tetraacetic acid (EGTA) ***(4)***. These indicators are polycarboxylate anions which are protected with ester groups, resulting in electrically neutral, lipophilic molecules that pass through the plasma membrane to the cytoplasm. The ester groups consist of acetate derivatives of aromatic alcohol (present in most fluorescent indicators) and acetoxymethyl ester of carboxylic acids (present in ion indicators). Intracellular esterases cleave the ester, liberating the more polar, polyanionic indicator in the cell ***(5)*** that can finally interact with the Ca^{2+} ions.

1.2. Meaurement of Ca^{2+} Throughout the Cells

Fura-2 is currently the most popular Ca^{2+} indicator for microscopy of individual cells. It retains the 1:1 stoichiometry, calcium selectivity, and pH insensitivity of the latter ***(6)***. The most important feature of fura-2 is the shift in excitation maximum upon binding Ca^{2+}. The excitation maxima of the calcium-free fura-2 is at 360 nm whereas the calcium-bound form excites at 340 nm. In the original method, fura-2 was used for producing and measuring

fluorescence at two excitation wavelengths and this allowed a concentration of calcium measurement based on the ratio of fluorescence at the two excitation wavelengths *(7)*. The method we use is based only on the mesurement at one excitation wavelength , namely of the calcium-bound form, 340nm. The green emission from fura-2 peaks at 505–520nm and does not shift with Ca^{2+} binding.

1.3. Calcium Calibration

The calibration procedure is simplified by applying the calibration formula developed by Grynkiewicz et al. *(6)* by using two extreme values for Ca^{2+} concentration (namely " zero" and "infinite" concentrations) and by using the original K_d determined in vitro which, for unknown reasons, has become the routinely used standard value under various experimental circumstances. The most commonly used procedure is the addition of ionophores. EGTA used at a concentration of 180 m*M* is added to the solution to chelate all free Ca^{2+} (F_{min}) and then HCl (1*M*) is added to lyse the cell. Consequently the dye is saturated with calcium (F_{max}).

2. Materials

2.1. Apparatus

1. Cell culture hood.
2. Incubator (37°C, 5% CO_2).
3. Ultrasonic water bath (Branson 1210, Merck).
4. Benchtop centrifuge.
5. Spectrofluorimeter (Jasco FP-777, Omnilab).
6. 1400 mm × 4 mm (320nm), round glass cuvets (ELMA, Hellma).
7. Magnet stirrer bar (6 mm × 3 mm).

2.2. Buffers

All buffers and solutions should be prepared from analytical grade reagents and dissolved in deionized water. The buffers can be stored at 4°C and are stable for 1 mo.

1. 1 m*M* fura-2-AM (Fluka, Buchs, Switzerland) dissolved in dimethyl sulfoxide (DMSO). Prepare aliquots that are only used once, and that are stored at –20°C in the absence of light.
2. PBS (1X): 0.14 *M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 10.1 m*M* Na_2HPO_4, adjust pH to 7.2 with 1 *M* NaOH.
3. Krebs Ringer Buffer
   a. Stock solutions: Solution 1: 0.6 *M* NaCl, 9.02 m*M* KCl, 5.9 m*M* KH_2PO_4, 5.9 m*M* $MgSO_4$. Solution 2: 31.24 m*M* $NaHCO_3$, 91.4 m*M* NaCl, 1.23m*M* EGTA. Add EGTA only on the day of the experiment. Solution 3: 1 *M* HEPES. Solution 4: 12 m*M* $CaCl_2$.

b. Mix the following amounts of the stock solution to prepare 1 L: 200 mL solution 1; 160 mL solution 2 (containing 1.23 m*M* EGTA added on the day of the experiment); 20 mL solution 3; 100 mL solution 4. Adjust the pH to 7.4 with 1 *M* NaOH, complete to 1 L with deionized water, and then add 1 g of D-Glucose.
c. On the day of the experiment, add to the solution 1 mg/mL BSA and 2 μL/mL fura-2 that has been sonicated for 5 min in an ultrasonic water bath. Protect the solution from light with aluminium foil and use it immediately to resuspend the cells.
d. The final ionic concentrations are: 136 m*M* NaCl, 1.8 m*M* KCl, 1.2 m*M* KH_2PO_4, 1.2 m*M* $MgSO_4$, 5.0 m*M* $NaHCO_3$, 1.2 m*M* $CaCl_2$, 0.2 m*M* EGTA, 5.5 m*M* D-glucose; 20 m*M* HEPES, 2 μ*M* Fura-2, 1m g/mL BSA.

4. Minimum buffer: 0.18 *M* EGTA; 1.40 *M* Tris-HCl; diluted 1:21 (v/v)digitonin.
5. Maximum buffer: 1.0 *M*HCl, 0.5 *M*$CaCl_2$.

3. Methods

3.1. Cell Preparation

The following method applies for adherent cells, suspended cells lines, or for purified leukocytes. Transfected mammalian cell lines such as CHO or HEK cells expressing recombinant receptors (*see* **Chapter 10**) are often used. For adherent cells, culture flasks of 175 cm^2 are normally used in order to obtain approx 2×10^7 cells. The same cellular concentration is used for purified leukocytes or suspended cells. On the day of the experiment, adherent cells should be maximally 80% confluent, but better results are obtained using 50 to 70% confluence.

1. Wash the cells twice with 10 mL of PBS.
2. Detach them with 10 mL PBS containing 1 m*M* EDTA and spin down 5 min at 296*g*.
3. Discard the supernatant.
4. Add 10–20 mL of culture medium and leave at 37°C under 5% CO_2 for 1–2 h.
5. Spin at 300*g* for 5 min, and remove the supernatant.
6. Resuspend the cells at a final density of approx 2×10^6 cells/mL in Krebs-Ringer Buffer containing BSA (1 mg/mL) and fura-2 (2 μL/mL).
7. Incubate 20–30 min at 37°C in absence of light.
8. Prepare 2 mL aliquots of the cell suspension in Eppendorf tubes and allow them to reach room temperature. Do not keep them at 37°C.
9. Treat one aliquot at a time. Centrifuge in a benchtop centrifuge (Eppendorf centrifuge) for 2 s to spin down the cells.
10. Resuspend in a suitable volume of Krebs-Ringer buffer to give a final cell density of 2×10^6 cells/mL. To keep this cell density with larger volume cuvets it is recommended to increase the number of cell flasks at the beginning of the experiment.
11. Transfer the cell suspension to preheated fluorescence cuvets.

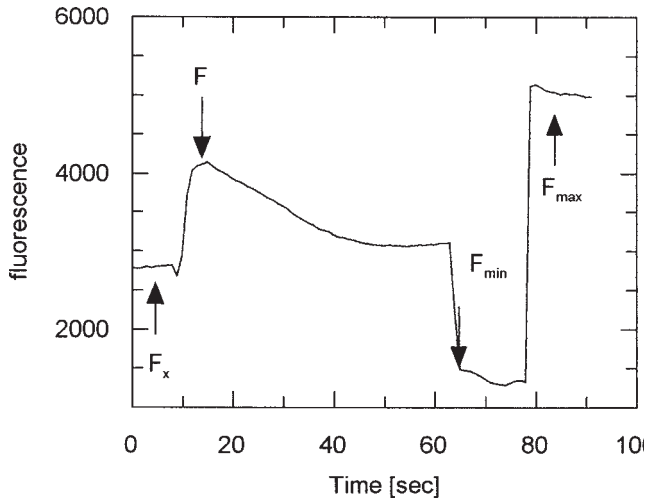

Fig. 1. Calcium mobilization on CHO cells expressing hCCR1 receptors.

3.2. Fluorescence Measurement

Measure the fluorescence emission at 505 nm using 340 nm excitation. Monitor the baseline. When a stable baseline is obtained for 10–30 s, add the chemokine to be tested. After the peak of calcium mobilization has subsided (about 1 min), calibrate the assay by adding 20 µL of minimum buffer, allow for stabilization, then add 20 µL of maximum buffer to dissolve cell membranes in order to obtain full complexation of fura-2 with Ca^{2+}. Record the baseline, the peak, the minimum and the maximum values (*see* **Fig. 1**). The concentration of Ca^{2+} mobilized is calculated according to the following modified formula ***(6)***:

$$[Ca^{2+}]\ (n\mathit{M}) = [Ca^{2+}_{max}] - [Ca^{2+}_{min}]$$

$$\text{where}\quad [Ca^{2+}_{min}] = K_d\,(F_x - F_{min})/(F_{max} - F_x) \qquad (1)$$

$$[Ca^{2+}_{max}] = K_d\,(F - F_{min})/(F_{max} - F)$$

$$\text{and } K_d = 224\ n\mathit{M}$$

F_x is the measured basal fluorescence, F, the measured cellular fluorescence, F_{max}, the fluorescence at saturing calcium concentration, and F_{min} the fluorescence at zero concentration.

References

1. Blinks, J. R., Wier, W. G., Hess, P., and Prendergast, F. G. (1982) Measurement of Ca^{2+} concentration in living cells. *Prog. Biophysics Mol. Biol.* **40,** 1–114.

2. Tsien, R. Y. and Rink, T. J. (1983) in *Current Methods in Cellular Neurobiolology* (Barker, J. L. and McKelvy, J. F., eds.) Vol III, John Wiley and Sons, New York, pp. 249–312.
3. Cobbold, P. H. and Rink, T. J. (1987) Fluorescence and bioluminescence measurement of cytoplasmic free calcium. *Biochem. J.* **248(2),** 313–328.
4. Tsien, R. Y. (1980) New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* **19,** 2396–2404.
5. Tsien, R. Y., Pozzan, T., and Rink, T. J. (1984) Calcium activities and fluxes inside small intact cells as measured with intracellularly trapped chelators. *Advances in cyclic nucleotide and protein phosphorylation Research* **17,** 535–541.
6. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260,** 3440–3450.
7. Tsien, R. Y., Rink, T. J., and Poenie, M. (1985) Measurement of cytosolic free Ca^{2+} in individual small cells using fluorescence microscopy with dual excitation wavelengths. *Cell Calcium* **6,** 145–157.

17

Actin Polymerization

Jörn Elsner and Alexander Kapp

1. Introduction

Locomotion of leukocytes is a complex event requiring coordinated activity of cytoskeletal, membrane, and adhesion systems *(1)*. Prior to the migration of cells into the inflamed tissue, they have to pass the endothelium. This process involves several types of adhesion molecule interactions between eosinophils and endothelial cells. Selectins mediate two steps, initial tethering to the vessel wall and their subsequent rolling. Integrins then bind to endothelial receptors, increasing adhesion which results in the arrest of the rolling leukocytes, which can then cross the endothelial layer of the blood vessel and enter the tissue using integrins for traction *(2–5)*.

Actin filaments are likely to be involved in multiple force-generating mechanisms. Forward motility of the membrane at the front of leading edge of the cell is called protrusion, and contains dense arrays of actin filaments. These filaments are organized with their barbed ends (actin filament growing or plus ends) in the direction of protrusion. An increase in the number of sites for actin polymerization is the first step. The polymerization and depolymerization of actin is regulated by actin-binding proteins. At micromolar and greater concentrations of calcium, and in the presence of low levels of the chemoattractant-induced phosphoinositides, the actin-binding protein gelsolin shortens the actin filaments and increases their number, but leaves them capped. At less than micromolar calcium, gelsolin dissociates from actin filaments, opening barbed ends for actin polymerization. Regulation of local free G-actin levels may be a primary effector for membrane extension. The amount of F-actin could potentially be increased by raising the concentration of G-actin monomers which exists in two pools, free G-actin and G-actin bound to a monomer-binding protein

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by β-thymosins (G-actin source), profilins (filament elongation promotor) and cofilins (filament cutter) ***(6,7)***.

Adhesion is required for protrusion to be converted into movement along the substrate. The process leading to forward movement of the cell is termed traction. The last step in locomotion is comprised of two mechanistically distinct processes: deadhesion (or detachment) and tail retration ***(6,7)***. Since the membrane protrusion has become adherent to the substratum forward translocation of the cell body may occur through myosin, another actin-binding protein through interactions with F-actin. Transient increases in intracellular calcium that occur during neutrophil migration regulate integrin-mediated adhesion to extracellular matrix proteins and are required for detachment of the rear cellular region and effective migration ***(8,9)***. Rapid migration requires efficient mechanisms to release adhesion at the rear of the cell. More recently, it has been demonstrated that extracellular matrix proteins, such as laminin and fibronectin, can attenuate both activation and degranulation of eosinophils and also influence their morphology after stimulation by C5a, PAF and IL-5 ***(10)***. In a previous study it was demonstrated that intracellular Ca^{2+} mobilisation in eosinophils following stimulation with C5a, PAF and RANTES represent an important step in the activation which leads to directed migratory responses and actin polymerization ***(11)***. These findings in eosinophils are in direct contrast to actin polymerization in neutrophils, in which intracellular calcium mobilization is not sufficient ***(12,13)***. Moreover, the cytoskeleton plays not only an important role in the process of cell migration but also in the regulation of the respiratory burst and enzyme release in human granulocytes ***(14,15)***.

One of the well established methods to investigate the dynamic process of actin polymerisation and depolymerisation in human granulocytes is the F-actin staining with 7-nitrobenz-2oxa-1,3-diazol (NBD)-phallacidin in flow cytometry ***(16,17)***. This compound, a fluorescent derivative of a group of bicyclic heptapeptides from poisonous mushrooms (phalloidin), binds with high affinity to F-actin but not G-actin. The fluorescence signal is a quantitative measure of the amount of F-actin in cells. In human granulocytes, maximal F-actin content is reached between 10–30 s after stimulation with chemokines and decreases to basal F-actin levels over 300 s. Since NBD-phallacidin does not permeate cell membranes, they need to be permeabilized with agents such as lysophosphtidylcholine ***(16,17)***.

2. Materials

1. Greiner mini tubes (Greiner, Germany, No. 101101).
2. FACScan (Becton Dickinson, Heidelberg, Germany).
3. Buffer A (Stop Buffer): Dilute 37% formaldehyde 1 : 5 with phosphate-buffered saline (PBS without Mg^{2+} and Ca^{2+}) (*see* **Note 1**).

4. Buffer B (NBD-phallacidin Buffer; 6.6×10^{-6} *M*) (*see* **Note 2**). Dissolve NBD-phallacidin (Molecular Probes, Amsterdam, NL, N-354) in 1.5 mL 99% methanol. Add 3.0 mL 37% formaldehyde. Add 10.5 mL PBS without Mg^{2+} and Ca^{2+}. Add 3.0 mg lysophosphatidylcholin (Sigma Chemicals, Deisenhofen, Germany, L-4129).

3. Methods

1. Purified granulocytes (eosinophils or neutrophils) are suspended at a concentration of 1×10^6 cells/mL in PBS without Mg^{2+} and Ca^{2+}.
2. Cells are preincubated at 37°C for 5 min.
3. 50 µL of Buffer A is added into the marked mini tubes (0s, 10s, 20s, 30s, 60s, 300s).
4. 50 µL of the cell suspension is added into 0s marked mini tube containing Buffer A.
5. The rest of the cell suspension is stimulated by the stimulus (e.g., eotaxin 100 ng/mL).
6. 50 µL of the stimulated cell suspension is added into the marked mini tubes containing Buffer A at different time points (10s, 20s, 30s, 60s, 300s).
7. Cell samples are kept at room temperature in the dark for 1 h.
8. 100 µL of Buffer B is added to each cell sample and incubated in the dark for 1 h.
9. Cell samples are analyzed on a Becton Dickinson FACScan with a linear fluorescence channel where the fluorescence is proportional to F-actin content. Samples were excited by an argon laser at 488 nm and emission was measured at 525 nm (green fluorescence/ FL1) ***(7)***.
10. Relative F-actin content is expressed as the ratio of the mean channel fluorescence (=integrated fluorescence) between stimulus-stimulated and nonstimulated cells ***(4,7,16)***.

4. Notes

1. This buffer should be kept at 4°C.
2. This buffer should be aliquoted in 1 mL tubes and kept at –20°C.

References

1. del Pozo, M. A., Sanchez-Mateos, P., and Sanchez-Madrid, F. (1996) Cellular polarization induced by chemokines: a mechanism for leukocyte recruitment. *Immunol. Today* **17,** 127–131.
2. Gumbiner, B. M. (1996) Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84,** 345–357.
3. Nakayama, H., Sano, H., Nishimura, T., Yoshidi, S., and Iwamoto, I. (1994) Role of vascular cell adhesion molecule 1/very late activation antigen 4 and intercellular adhesion molecule 1/lymphocyte function-associated antigen 1 interactions in antigen-induced eosinophil and T cell recruitment into the tissue. *J. Exp. Med.* **179,** 1145–1154.

4. Springer, T. A. (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* **76,** 301–314.
5. Symon, F. A., Walsh, G. M., Watson, S. R., and Wardlaw, A. J. (1994) Eosinophil adhesion to nasal polyp endothelium is P-selectin-dependent. *J. Exp. Med.* **180,** 371–376.
6. Lauffenburger, D. A. and Horwitz, A. F. (1996) Cell migration: a physically integrated molecular process. *Cell* **84,** 359–369.
7. Mitchison, T. J. and Cramer, L. P. (1996) Actin-based cell motility and cell locomotion. *Cell* **84,** 371–379.
8. Hendey, B., Lawson, M., Marcantonio, E. E., and Maxfield, F. R. (1996) Intracellular calcium and calcineurin regulate neutrophil motility on vitronectin through a receptor identified by antibodies to integrins αv and β3. *Blood* **87,** 2038–2048.
9. Lawson, M. A. and Maxfield, F. R. (1995) Ca^{2+}- and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils. *Nature* **377,** 75–79.
10. Kita, H., Horie, S., and Gleich, G. J. (1996) Extracellular matrix proteins attenuate activation and degranulation of stimulated eosinophils. *J. Immunol.* **156,** 1174–1181.
11. Elsner, J., Dichmann, S., Dobos, G. J., and Kapp, A. (1996) Actin polymerization in human eosinophils, unlike human neutrophils, depends on intracellular calcium mobilization. *J. Cell Physiol.* **167,** 548–555.
12. Downey, G. P., Chan, C. K., Trudel, S., and Grinstein, S. (1990) Actin assembly in electropermeabilized neutrophils: Role of intracellular calcium. *J. Cell. Biol.* **110,** 1975–1982.
13. Salmon, J. E., Brogle, N. L., Edberg, J. C., and Kimberly, R. P. (1991) Fc receptor III induces actin polymerization in human neutrophils and primes phagocytosis mediated by Fcg receptor II. *J. Immunol.* **146,** 997–1004.
14. Bengtsson, T., Zalavary, S., Stendahl, O., and Grenegard, M. (1996) Release of oxygen metabolites from chemoattractant-stimulated neutrophils is inhibited by resting platelets: Role of extracellular adenosine and actin polymerization. *Blood* **10,** 4411–4423.
15. Wiles, M. E., Dykens, J. A., and Wright, C. D. (1995) Human neutrophil (PMN) oxygen radical production and the cytoskeleton. *Life Sci.* **57,** 1533–1546.
16. Howard, T. H. and Meyer, W. H. (1984) Chemotactic peptide modulation of actin assembly and locomotion in neutrophils. *J. Cell Biol.* **98,** 1265–1271.
17. Wallace, P. J., Wersto, R. P., Packman, C. H., and Lichtman, M. A. (1984) Chemotactic peptide-induced changes in neutrophil actin conformation. *J. Cell. Biol.* **99,** 1060–1065.

18

Reactive Oxygen Release

Jörn Elsner and Alexander Kapp

1. Introduction

Monocytes, macrophages, neutrophils, and eosinophils are able to generate and release reactive oxygen species. The reactive oxygen species are generated by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which is activated by a number of different soluble and particulate agents. This activation results in the reduction of molecular oxygen to the potentially toxic oxygen species superoxide anion (O_2^-), or hydrogen peroxide (H_2O_2), with NADPH serving as the electron donor. In the presence of eosinophil peroxidase, H_2O_2 may then give rise to the potentially cytotoxic hypohalous acids such as HOBr. The increase in oxygen consumption is termed the respiratory burst ***(1,2)***.

One of the major bactericidal mechanisms of granulocytes such as neutrophils involves activation of the respiratory burst to generate reactive oxygen species. The oxidase is rapidly activated, often within a minute, in response to extracellular signals such as chemotaxins, inflammatory mediators, and invading microorganisms ***(3)***. Tissue damage and propagation of inflammation in allergic diseases such as allergic asthma and atopic dermatitis is thought to be mediated by the interaction between Th2-like T cells, antigen-presenting cells, and eosinophils ***(4–7)***. In this process, eosinophils are activated by several cytokines and inflammatory mediators leading to tissue injury by the release of toxic granule proteins and generation of reactive oxygen species ***(8–12)***. The reactive oxygen species are generated by the NADPH oxidase that can be activated by a number of different soluble and particulate agents, such as PMA, IL-3, IL-5, GM-CSF, C5a, PAF, eotaxin, and MCP-3 ***(13–16)***.

The phorbolester phorbol myristate acetate (PMA), which directly activates protein kinase C by bypassing receptor-coupled G-proteins, is the most potent stimulus that produces reactive oxygen species in human eosinophils. This sug-

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gests that protein kinase C plays a key role in the activation of the respiratory burst ***(9)***. This hypothesis is further supported by the inhibition of the respiratory burst in eosinophils by the protein kinase C inhibitor staurosporin ***(9,15,17)***. Besides protein kinase C, other protein kinases have been investigated in the involvement of the respiratory burst in human granulocytes. There is increasing evidence that 1-phosphatidylinositol 3-kinase and tyrosine kinases are also involved in the regulation of the respiratory burst ***(18,19)***. Moreover, in a recent study it was demonstrated that intracellular Ca^{2+} mobilization in eosinophils is an important step in the activation of the respiratory burst following stimulation with chemotaxins ***(9)***.

There are many methods to investigate the release of reactive oxygen species in human granulocytes. One of the most established methods is the assessment of superoxide anion (O_2^-) production which is quantified as the superoxide dismutase inhibitable reduction of ferricytochrome C ***(13)***. A disadvantage of this assay is the large number of cells in each sample (1×10^6 cells/mL) which is the limiting factor to investigate eosinophils from normal nonatopic donors. Another well established assay is the measurement of intracellular hydrogen peroxide (H_2O_2) with dihydrorhodamine 123 which is oxidized by H_2O_2 during the respiratory burst, to brightly fluorescent rhodamine 123 which can be analyzed by flow cytometry ***(20)***. Another well established technique to investigate the release of reactive oxygen species is the lucigenin-dependent chemiluminescence (CL). This method is very sensitive and assesses the extracellular release of reactive oxygen species ***(21,22)*** which is mainly dependent on the release of the superoxide anion since more than 80% of the signal is inhibited by superoxide dismutase ***(23)***. A further advantage is the measurement of many samples simultaneously in a 96-wells format and the low number of cells required for each sample (5,000 eosinophils and 50,000 neutrophils) ***(20,24)***.

2. Materials

1. Dilute 120 mg Lucigenin (Sigma Chemical, Deisenhofen, Germany; M-8010) in 20 mL HEPES- buffered Hanks' balanced salt solution (HBSS; Gibco, Grand Island, NY). Lucigenin solution should kept at 4°C.
2. Flat-bottom white microtiter plates (Microfluor, Dynatech, Denkendorf, Germany, No. 7905).
3. Single-photon imaging system with a two-dimensional photon counting system (MTP reader, Hamamatsu Photonics, Herrsching, Germany).

3. Methods

1. Purified eosinophils and neutrophils are suspended at 5×10^4 cells/mL and 5×10^5 cells/mL, respectively, in HEPES- buffered Hanks' balanced salt solution, pH 7.4 containing 1 mg/mL BSA (HBSS/BSA).
2. Cells are diluted 1:60 with lucigenin solution (200 μ*M* lucigenin).

3. 100 μL of the lucigenin cell suspension is added into the white flat microtiter plate and incubated for 10 min at 4°C.
4. The microtiter plate containing lucigenin loaded cells is placed into the single-photon imaging system with a two-dimensional photon counting system microtiter plate ([MTP]-reader) and the prerun started for 30 min at 37°C.
5. The microtiter plate is removed from the MTP-reader and cells stimulated very quickly by different stimuli and medium (25 μL), respectively.
6. The microtiter plate is rapidly inserted into the MTP-reader and the run restarted for 60 min at 37°C.
7. Measurements are performed in triplicate at 37°C.
8. Integral counts from 0–60 min incubation intervals after addition of the stimuli to the cell sample are measured and expressed as intensity integral counts or as the ratio between stimuli to medium stimulated cells.

References

1. Lundqvist, H., Follin, P., Khalfan, L., and Dahlgren, C. (1996) Phorbol myristate acetate—induces NADPH oxidase activity in human neutrophils: only half the story has been told. *J. Leukoc. Biol.* **59,** 270–279.
2. Remick, D. G. and Villarete, L. (1996) Regulation of cytokine gene expression by reative oxygen and reactive nitrogen intermediates. *J. Leukoc. Biol.* **59,** 471–475.
3. Lambeth, J. D. (1988) Activation of the respiratory burst oxidase in neutrophils: on the role of membrane-derived second messaengers, Ca^{2+}, and protein kinase. *C. J. Bioenerg. Biomemb.* **20,** 709–733.
4. Bieber, T., de la Salle, H., Wollenberg, A., Hakimi, J., Chizzonite, R., Ring, J., Hanau, D., and de la Salle, C. (1993) Human epidermal Langerhans cells express the high affinity receptor for immunoglobulin E (Fc epsilon RI). *J. Exp. Med.* **175,** 1285–1290.
5. Busse, W. W. and Sedgwick, J. B. (1992) Eosinophils in asthma. *Ann. Allergy* **286,** 286–290.
6. Gleich, G. J., Adolphoson, C. R., and Leifermann, K. M. (1993) The biology of the eosinophilic eosinophilic leukocyte. *Annu. Rev. Med.* **44,** 85–101.
7. Romagnani, S. (1997) The Th1/Th2 paradigm. *Immunol. Today* **18,** 263–266.
8. Coyle, A. J., Ackermann, S., Burch, R., Proud, D., and Irvin, C. G. (1995) Human eosinophil granule major basic protein and synthetic polycations induce airway hyperresponsiveness in vivo dependent on bradykinin generation. *J. Clin. Invest.* **95,** 1735–1740.
9. Elsner, J., Dichmann, S., and Kapp, A. (1995) Activation of the respiratory burst in human eosinophils by chemotaxins requires intracellular calcium fluxes. *J. Invest. Dermatol.* **105,** 231–236.
10. Kapp, A. (1993) The role of eosinophils in the pathogenesis of atopic dermatitis—eosinophil granule proteins as markers of disease activity. *Allergy* **48,** 1–5.
11. Rochester, C. L., Ackermann, S. J., Zheng, T., and Elias, J. A. (1996) Eosinophil-fibroblast interactions—granule major basic protein interacts with IL-1 and transforming growth factor-beta in the stimulation of lung fibroblast IL-6-type cytokine production. *J. Immunol.* **156,** 4449–4456.

12. Rosenberg, H. F. and Tiffany, H. L. (1994) Characterization of the eosinophil granule proteins recognized by the activation-specific antibody EG2. *J. Leukoc. Biol.* **56,** 502–506.
13. Elsner, J., Hochstetter, R., Kimmig, D., and Kapp, A. (1996) Human eotaxin represents a potent activator of the respiratory burst of human eosinophils. *Eur. J. Immunol.* **26,** 1919–1925.
14. Elsner, J., Oppermann, M., Czech, W., Dobos, G., Schöpf, E., Norgauer, J., and Kapp, A. (1994) C3a activates reactive oxygen radical species production and intracellular calcium transients in human eosinophils. *Eur. J. Immunol.* **24,** 518–522.
15. Kapp, A., Zeck Kapp, G., Czech, W., and Schöpf, E. (1994) The chemokine RANTES is more than a chemoattractant: Characterization of its effect on human eosinophil oxidative metabolism and morphology in comparison with IL-5 and GM-CSF. *J. Invest. Dermatol.* **102,** 906–914.
16. Zeck Kapp, G., Kroegel, C., Riede, U. N., and Kapp, A. (1995) Mechanisms of human eosinophil activation by complement protein C5a and platelet-activating factor: similar functional responses are accompanied by different morphologic alterations. *Allergy* **50,** 34–47.
17. Kernen, P., Wymann, M. P., von Tscharner, V., Deranleau, D. A., Tai, P. C., Spry, C. J., Dahinden, C. A., and Baggiolini, M. (1991) Shape changes, exocytosis, and cytosolic free calcium changes in stimulated eosinophils. *J. Clin. Invest.* **87,** 2012–2017.
18. Nagata, M., Sedgwick, J. B., Bates, M. E., Kita, H., and Busse, W. W. (1995) Eosinophil adhesion to vascular cell adhesion molecule-1 activates superoxide anion generation. *J. Immunol.* **155,** 2194–2202.
19. Thelen, M., Wymann, M. P., and Langen, H. (1994) Wortmannin binds specifically to 1-phosphatidylinositol 3-kinase while inhibiting guanine nucleotide-binding protein-coupled receptor signaling in neutrophil leukocytes. *Proc. Natl. Acad. Sci. USA* **91,** 4960–4964.
20. Elsner, J., Oppermann, M., Czech, W., and Kapp, A. (1994) C3a activates the respiratory burst in human polymorphonuclear neutrophilic leukocytes via pertussis toxin-sensitive G-proteins. *Blood* **83,** 3324–3331.
21. Maly, F. E., Urwyler, A., Rolli, H. P., Dahinden, C., and de Weck, A. L. (1988) A single-photon imaging system for the simultaneous quantitation of luminescent emissions from multiple samples. *Anal. Biochem.* **168,** 462–469.
22. Wymann, M. P., Kernen, P., Deranleau, D. A., and Baggiolini, M. (1989) Respiratory burst oscillations in human neutrophils ad their correlation with fluctuations in apparent cell shape. *J. Biol. Chem.* **264,** 15,829–15,834.
23. Kapp, A., Zeck Kapp, G., Danner, M., and Luger, T. A. (1988) Human granulocyte-macrophage colony stimulating factor: An effective direct activator of human polymorphonuclear neutrophilic granulocytes. *J. Invest. Dermatol.* **91,** 49–55.
24. Elsner, J., Oppermann, M., and Kapp, A. (1996) Detection of C5a receptors on human eosinophils and inhibition of eosinophil effector functions by anti-C5a receptor (CD88) antibodies. *Eur. J. Immunol.* **26,** 1560–1564.

19

Histamine Release

Michael A. Lett-Brown and Rafeul Alam

1. Introduction

Basophils and mast cells play an important role in allergic disorders. Both of these cell types produce and secrete many mediators that play an obligatory role in immediate hypersensitivity reactions. Some of the mediators are preformed and stored in granules. An example is histamine. Other mediators are generated *de novo*, e.g., eicosanoids. Mast cells and basophils produce some important cytokines, e.g., IL-4, IL-13, TNF-α ***(1–5)*** and the chemokines MIP-1α and RANTES ***(6)*** upon activation. The cells are typically activated by cross-linking the high affinity IgE receptors. In addition to this classical activation mechanism basophils respond to a wide variety of stimuli. They include C5a, C3a, FMLP, hyperosmolar solutions, neuropeptides, histamine releasing factor, and CC chemokines ***(7)***. The most potent basophil-activating CC chemokines are MCP-1, MCP-3, MCP-4, eotaxin, RANTES, and MIP-1α ***(7)***.

Of their many preformed intracellular mediators, histamine, not only plays an important physiological role, but can be used as a "marker" for these cells. In the human peripheral blood, the basophil is the only leukocyte that contains histamine. As a result, histamine release from human blood leukocyte preparations can be used to monitor basophil degranulation. In this chapter we will describe the histamine release assay, alternative techniques to measure the concentration of histamine and, finally, a method to purify human basophils to confirm that the chemokines are acting directly on the basophil.

2. Materials

2.1. Purification of Basophils

1. Percoll Gradient Preparation: Percoll Stock: Mix 180 mL Percoll (Pharmacia, Piscataway, NJ), 18 mL 10X Hanks' Balanced Salt Solution (HBSS) without Ca,

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Mg, phenol red, or bicarbonate (Gibco-BRL), 2 mL pH 7.3, 0.25 *M* HEPES buffer (Gibco-BRL), and 0.8 mL 1 *N* HCl. The pH of the solution should be 7.4.

2. Percoll solutions of different density:

Density (g/mL)	Percoll stock/1X HBSS (mL/mL)
1.070	24.0 : 20.0
1.079	27.0 : 15.9

We verify the density using a sterile hydrometer. A more accurate technique, used by Leonard et al. ***(8)***, is to measure the refractive index (RI) which is related linearly to the density. The slope of the straight line can be determined by carefully weighing 25 mL vol of 3 different solutions and measuring the RI at 22°C. A change of density of 0.001 corresponds to a change of 1.5×10^{-4} in RI. Final adjustments of density are made with HBSS (to reduce density) or Percoll stock (to increase density).

2.2. Histamine Release Assay: Stock Solutions

1. 6% Hydroxyethyl Starch.
2. 10X HEPES buffer: 7.44 g KCl + 47.6 g HEPES + 16 g NaCl dissolved in 1800 mL distilled water, adjust pH to 7.6 with 10 *N* NaOH. Bring up to 2 L with distilled water. Conductivity = 90,000 OHMS (stable at 4°C for at least 2 mo).
3. Human Serum Albumin (HSA, Siga, Fraction V Powder): 3.0 g in 100 mL distilled water, prepare 3 mL aliquots and store at –20°C.
4. Divalent cations (0.2 *M* Ca^{2+}, 0.1*M* Mg^{2+}): 29.4 g $CaCl_2 \cdot 2H_2O$ + 20.3g $MgCl_2 \cdot 6H_2O$ in 1 L of distilled water. Conductivity = 35,000 OHMS pH = 7.0. (Stable at 4°C for at least 2 mo.)
5. EDTA: 37.2 g EDTA in 800 mL distilled water, titrate to pH 7.2 with 10 *N* NaOH, bring up vol to 1 L with distilled water. Store at 4°C. Conductivity = 17,000 OHMS. (Stable at 4°C for at least 2 mo.)

   On the day of an experiment prepare the following solutions from the above stock solutions:

   HA: To 20 mL of 10X HEPES buffer add 2 mL of thawed 3% HSA. Bring vol up to 200 mL in a volumetric flask.

   HACM: To 10 mL of 10X HEPES buffer add 1 mL of 3% human serum albumin (HSA) and 1 mL of divalent cation solution. Bring vol up to 100 mL in a volumetric flask.

These solutions should be discarded at the end of the experiment.

3. Methods

3.1. Purification of Basophils

The technique presented here is a minor modification ***(9)*** of that described by Leonard et al. ***(8)***.

1. To 15 mL polystyrene conical tubes add 6 mL of isotonic Percoll (density, 1.070g/mL). Underlayer with 3 mL isotonic Percoll (density, 1.079g/mL). We use a Gilson Minipuls II pump with tubing narrowed at the end which allows us to prepare 5 tubes at a time.
2. Carefully layer 5 mL of peripheral blood anticoagulated with 0.01 *M* EDTA above the Percoll gradient.
3. Centrifuge the gradients at 300*g* for 25 min at room temperature. To avoid gradient pertubations, disconnect the centrifuge brake.
4. Enriched preparations of basophils will form in a band between the two Percoll layers. Carefully remove the upper band using a 1 mL disposable polypropylene transfer pipet.
5. Using a fresh pipet, remove the second band containing the majority of the basophils. Dilute the cell suspension with HBSS containing 1% BSA. Centrifuge at 300*g* for 10 min at 4°C, discard the supernatant and repeat the wash with HBSS/1% BSA. Resuspend cells in 2 mL HBSS/1% BSA. Usually these preparations will contain 20–60% basophils (based on Wright-Giemsa stain of cytocentrifuge preparations).
6. If further purification is required add antibody-coated (anti-CD2,-CD14, and -CD19) magnetic beads (Dynabeads M-450) at a ratio of 10 beads/cell. Incubate at 4°C for 30 min with gentle rocking.
7. The 5 mL tube is placed in a magnetic field (DYNAL MDC 1) for 2 min, the supernatant is gently removed with a 1 mL disposable polypropylene transfer pipette. Resuspend the beads in 2 mL HBSS/1% BSA and place the tube in the magnetic field again for 2 min. Collect the supernatant, combine the 2 supernatants and centrifuge at 300*g* for 10 min. Resuspend the cells in the desired volume of HACM.
8. This method of purification yields 20–50% of the total peripheral blood basophils with a purity of 60–90%. Other methods of basophil purification have previously been described. One such method involves Percoll gradient centrifugation followed by selection of IgE+ cells by passing them through an allergen (penicillin)-containing column ***(10)***. This method yields 28–64% of the total peripheral blood basophils of 92% purity.

3.2. Histamine Release

Histamine release by chemokines can be tested using either purified basophils or total leukocyte preparations ***(11)***. The preparation of purified basophils is described above. In this section we will first describe the preparation of peripheral blood leukocytes and then present the histamine release test.

3.2.1. Isolation of Peripheral Blood Leukocytes

The volume of blood required is dependent on the number of samples to be tested. We usually use 0.5–1 mL of blood per reaction tube (e.g., for an experiment involving 50 samples we use approx 30 mL of blood).

1. Add 7.5 mL of 6% hydroxyethyl starch to a 50 mL conical polypropyline tube and 1/10 the blood vol of 0.1 *M* EDTA.
2. Following venous blood collection into a plastic syringe, add 25–35 mL of blood to the tube prepared in **step 1**.) Recap the tube and mix by inverting 4–5 times. Loosen the cap to prevent small volumes of blood collecting at the top of the tube. Allow to stand at room temperature for approx 30 min until there is a clear separation between the upper leukocyte rich buffy coat layer and the red cell layer.
3. Carefully collect the upper buffy coat layer with a plastic pipet without disturbing the red cell layer.
4. Centrifuge the leukocyte-rich buffy coat layer at 300*g* for 15 min at 4°C. Carefully decant the supernatant and resuspend the cell pellet by tapping the bottom of the tube. Resuspend the cells in approximately their original buffy coat volume in HA buffer at 4°C. Centrifuge at 300*g* for 10 min at 4°C. Repeat the washing procedure twice and finally resuspend the cells in the desired volume of HACM at 37°C (50 µL × number of samples + 10% of this total volume).

3.2.2. Histamine Release Assay

1. While the blood is sedimenting prepare the reaction tubes. Add 50 µL of 6% perchloric acid to four 1.5 mL microcentrifuge tubes (to determine total histamine content of the cells using the automated fluorometric analyzer). Add 50 µL of HACM to three 1.5 mL microcentrifuge tubes (to determine the spontaneous release of histamine). To a series of tubes add 50 µL of different dilutions of the chemokines; duplicate or triplicate samples should be used. The concentration of the chemokines should range between 2×10^{-11} *M* and 2×10^{-6} *M*, using 10-fold dilutions. Triplicate samples of a positive control rabbit antihuman IgE (1 : 3000 dilution) should routinely be used.
2. Allow the reaction tubes to equilibrate for 10 min in a 37° C shaking water bath. Then add 50 µL of the washed leukocyte suspension in HACM at 37°C to each reaction tube. Incubate in the shaking water bath at 37°C for 45 min. Then add 400 µL of HA buffer to each tube at the end of the incubation. Centrifuge the tubes at 600*g* for 10 min at 4°C. Separate the supernatants for measurement of histamine.

The histamine content of the supernatants can be determined using the automated fluorometric analyzer ***(12)***. Other methods used include a radioisotopic-enzymatic assay ***(13)*** or an enzyme immunoassay ***(14)***.

4. Notes

4.1. Purification of Basophils

Success in purifying peripheral blood basophils depends on a number of factors:

1. It is our experience that donors must be carefully selected; as shown by Leonard et al. ***(8)***, a relatively high percentage of basophils from a few donors have a

density of >1.070 and will form a band between the plasma and upper Percoll layer (d = 1.070) where the majority of lymphocytes and monocytes form a band. Because of this problem we exclude these donors.
2. It is essential to carefully remove the upper thick band of cells consisting mainly of lymphocytes and monocytes in order to prevent contamination of the basophil enriched lower band between the two Percoll layers.
3. The difference in the density of the two Percoll solutions is ~1% so small differences will affect the results. We have also noted that leaving the blood at room temperature for any extended time before adding it to the gradient causes basophils to localize in the lymphocyte/monocyte layer. To prevent this problem, we collect the blood into EDTA (final concentration 0.01 *M*) after we have prepared the required number of tubes containing the two Percoll layers.

4.2. Histamine Release

4. It has been our experience that determining both the pH and the conductivity of both our stock solutions and the solutions used on the day of an experiment ensures quality control. Stock solutions should be discarded if any cloudiness or precipitation is noticed. Since degranulation of basophils is dependent on divalent cations, omission of these cations in the preparation of HACM will result in low release of histamine.

References

1. Plaut, M., Pierce, L. H., Watson, C. J., Hanley-Hyde, L., Nordan, R. P., and Paul, W. E. (1989) Mast cell lines produce lymphokines in response to cross-linkage of Fc ε RI or calcium ionophores. *Nature* **339,** 64–67.
2. Gordon, L. R., Burd, P. R., and Galli, S. J. (1990) Mast cells as a source of multifunctional cytokines. *Immunol. Today* **11,** 458–464.
3. Lett-Brown, M. A., Li, H., Stafford, S., Hilsmeier, K., Sim, T., Grant, J. A., and Alam, R. (1993) Human basophils produce IL-4 in response to anti-IgE. *J. Allergy Clin. Immunol.* **91,** 257 (Abstract).
4. Brunner, T., Heusser, C. H., and Dahinden, C. A. (1993) Human peripheral blood basophils primed by IL-3 produce IL-4 in response to immunoglobulin E receptor stimulation. *J. Exp. Med.* **177,** 605–611.
5. Li, H., Sim, T. C., and Alam, R. (1995) IL-13 is released by and localized in human basophils. *J. Immunol.* **156,** 4833–4838.
6. Li, H., Sim T. C., Grant, J. A., and Alam, R. (1996) The production of MIP-1α by human basophils. *J. Immunol.* **157,** 1207–1212.
7. Alam, R. and Grant, J. A. (1995) The chemokines and the histamine releasing factors: modulation of function of basophils, mast cells and eosinophils, in *Human Basophils and Mast Cells: Biological Aspects* (Marone, G., ed.), Karger, Basel, pp. 148–160.
8. Leonard, E. J., Roberts, R. L., and Skeel, A. (1984) Purification of human blood basophils by single step isopycnic banding on Percoll. *J. Leukocyte Biol.* **35,** 169–177.

9. Lett-Brown, M. A., Robinson, L., Juneja, H. S., and Grant, J. A. (1989) Purification of human basophils: their response to anti-IgE. *J. Immunol. Methods* **117,** 163–167.
10. Warner, J. A., Reshef, A., and MacGlashan, D. W. Jr. (1987) A rapid Percoll technique for the purification of human basophils. *J. Immunol. Methods* **105,** 107–110.
11. Alam, R., Forsythe, P. A., Stafford, S., Lett-Brown, M. A., and Grant, J. A. (1992) Macrophage inflammatory protein-1α activates basophils and mast cells. *J. Exp. Med.* **176,** 781–786.
12. Siraganian, R. P. (1975) Refinements in the automated fluorometric histamine analysis system. *J. Immunol. Methods* **7,** 283–289.
13. Shaffe, R. E. and Beaven, M. A. (1979) Increased sensitivity of the enzymatic isotopic assay of histamine: measurement of histamine in plasma and serum. *Anal. Biochem.* **94,** 425–430.
14. Chevrier, D. C., Guesdon, J.-L., Mazie, J.-C., and Avrameas, S. (1986) Enzyme immunoassay for the measurement of histamine. *J. Immunol. Methods* **94,** 119–125.

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Measurement of Phosphoinositide 3-Kinase Activity

Stephen G. Ward

1. Introduction

The term "PI 3-kinase" is now applied to a growing family of proteins that are able to convert phosphatidylinositol (PtdIns), PtdIns(4)P and PtdIns(4,5)P_2 into PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 respectively, by phosphorylating the D-3 position of the inositol head groups of phosphoinositide lipids (collectively known as D-3 phosphoinositide lipids shown in **Fig. 1**) ***(1,2)***. PtdIns(3)P is constitutively present in eukaryotic cells and its levels are largely unaltered upon cellular stimulation. In contrast, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are generally absent from resting cells, but their intracellular concentration rises markedly upon stimulation via a variety of receptors suggesting a likely function as a second messenger ***(1,2)***. PI 3-kinases can be divided into three main classes on the basis of their in vitro lipid substrate specificity, structure and likely mode of regulation. Class I PI 3-kinases phosphorylate PtdIns, PtdIns(4)P and PtdIns(4,5)P_2, interact with Ras and form heterodimeric complexes with adaptor proteins that link them to different upstream signaling events ***(1)***. The prototypical class IA PI 3-kinase is a heterodimer consisting of the 85 kDa regulatory subunit (responsible for protein-protein interactions either via protein tyrosine phosphate-binding SH2 domains or SH3 domains and/or proline rich regions) and a catalytic 110 kDa subunit. The class IB PI 3-kinases are stimulated by G protein βγ subunits and do not interact with the SH2-containing adaptors that bind class IA PI 3-kinases. Instead, the first identified member of this family p110γ, associates with a unique p101 adaptor molecule ***(1)***. The class II PI 3-kinases are characterized by the presence of a C-2 domain at the carboxy terminus and utilize predominantly PtdIns and PtdIns(4)P as substrates (e.g., PI3K-C2α), whereas

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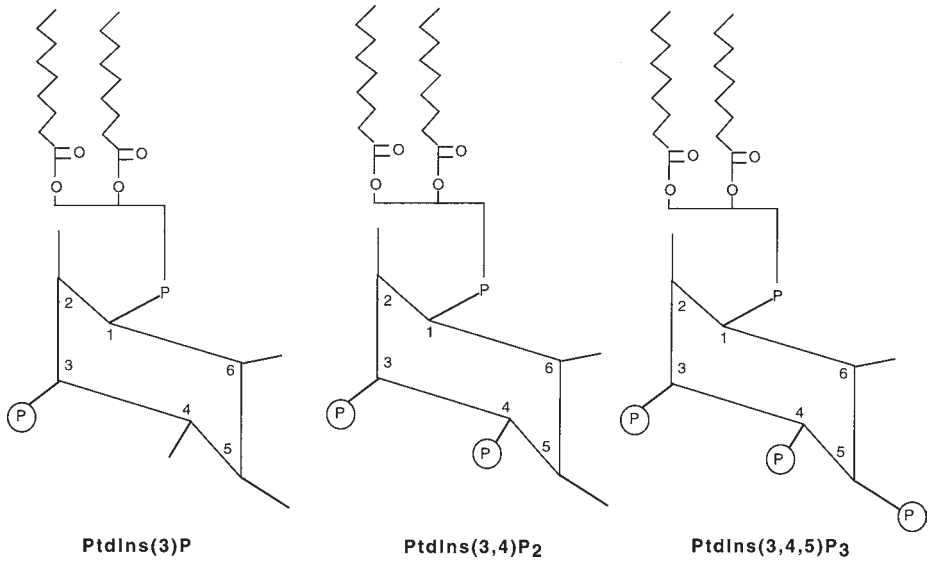

Fig. 1. Schematic representations of D-phosphatidylinositol 3-phosphate [PtdIns(3)*P*], D-phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P_2] and D-phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3].

the class III PI 3-kinases utilize only PtdIns as a substrate (e.g., mammalian PtdIns 3-kinase and yeast Vps34p) ***(1)***. Generally, PI 3 kinases are now regarded as an important intracellular signal upstream of a variety of biochemical (e.g., activation of Akt/protein kinase B and/or p70 S6 kinase and inhibition of glycogen synthase kinase-3) and functional responses (membrane trafficking of proteins such as the glucose transporter GLUT4, postendocytic sorting of ligand-stimulated receptors such as the Platelet-derived growth factor (PDGF) receptor, membrane ruffling, superoxide production, and chemotaxis) ***(1,2)***.

There are two main procedures for measuring PI 3-kinase activity which measure lipid kinase activity in intact cells or broken cell lysates respectively, and both rely on detecting the transfer of the γ- phosphate of ATP to the D-3 position of the inositol head group of phosphoinositide lipids. The first method relies on metabolic labeling of intact cellular pools of ATP with [^{32}P]Pi followed by lipid extraction ***(3,4)*** and separation of the phosphorylated lipids by high-performance liquid chromatography (HPLC) analysis ***(5)***. The advantages of this procedure are:

1. Allows quantitative detection of all phosphoinositide lipids with good resolution. Thus, one sample can provide a large amount of information concerning receptor-induced effects on all the phosphoinositide lipids.

2. Very sensitive detection of D-3 phosphoinositides, which generally form less than 0.25% of the total phosphoinositide lipid pool.

However, there are also several disadvantages to this procedure:

1. It requires the use of mCi amounts of [^{32}P]Pi and can therefore constitute a considerable radiochemical hazard.
2. Requires the use of specialized HPLC apparatus connected to a fraction collector or an on-line β-radiodetector.
3. Does not necessarily distinguish between the lipid kinase activity of various different isoforms and subtypes of the PI 3-kinase family.
4. Time consuming, expensive, and generates a lot of liquid radioactive waste.

In the second procedure, it is possible to assay specific immunoprecipitated proteins (e.g., PI 3-kinase subunits, receptors, receptor component chains, or even cellular proteins) for associated lipid kinase activity under in vitro assay conditions using distinct substrates such as PtdIns ***(6–8)***. It is also possible to use PtdIns(4)P or PtdIns(4,5)P_2 as in vitro substrates for the prototypical class IA PI 3-kinase, but generally these are more expensive to buy.

The advantages of this procedure are:

1. Can distinguish between various PI 3-kinase subtypes by varying substrate and adding reagents to assay buffer that differentially affect PI 3-kinase substrates.
2. Less radiochemical hazard than that associated with the first procedure.
3. Cheaper and quicker to perform than the first procedure.
4. Does not require expensive specialized apparatus.

The disadvantages of this procedure are:

1. Regulatory properties and precise substrate specificities of the PI 3-kinase(s) may be remarkably distorted by the assay conditions and form of lipid presentation used.
2. Less sensitive than the first procedure.
3. May detect other lipid kinases such as PI 4-kinases that coassociate with immunoprecipitated proteins or nonspecifically associate with protein A-Sepharose beads.
4. Requires separation of extracted lipids by thin layer chromatography (TLC) which poorly resolves specific D-3 phosphoinositides such as PtdIns(3)P from nonspecific products such as PtdIns(4)P.
5. Quantitation requires a phosphoimager which may not be readily available. Alternatively, the relevant regions of the silica gel may be scraped from the TLC into scintillation liquid followed by β-scintillation counting, but this is tedious and can lead to loss of material and is consequently inaccurate.

2. Materials

2.1. Measurement of D-3 Phosphoinositide Lipids

1. The following solutions and organic solvent mixtures should be prepared from analytical grade reagents:

a. Sterile phosphate-free balanced salt solution supplemented with sodium bicarbonate and 20 m*M* HEPES, adjusted to pH 7.4 with 10 *M* NaOH. Store at 4°C for up to 1 mo (*see* **Note 1**).
b. Fetal calf serum (FCS) that has been dialysed overnight against saline to remove any phosphate from the FCS. Should be sterile filtered and stored in aliquots at –20°C. Working aliquots can be stored at 4°C for 1 mo.
c. 100 mL chloroform/methanol/H_2O (32.6:65.3:2.1% v:v:v).
d. Chloroform containing 10 μg/mL Folch lipids. Make up fresh just before use.
e. 2.4 *M* HCl, 5 m*M* tetrabutylammonium sulphate. Make up fresh just before use.
f. 25% methylamine in H_2O/methanol/N-butanol (4:4:1 v/v).
g. N-butanol/petroleum ether (bp 40–60°C)/ethyl formate (20:4:1 v/v).

2. 1.25 *M* $(NH_4)_2HPO_4$ adjusted to pH 3.8 with H_3PO_4. This buffer should be filtered through Whatman 2 μm cellulose nitrate filters before use. Solution is stored at room temperature and is stable for up to a month. Solution should however, be degassed by refiltering and bubbling through with N_2 for 30 min before it is used.
3. [^{32}P]-Pi (8500-9120 Ci/mmol), [^{3}H]-phosphatidylinositol (1-20 Ci/mmol), [^{3}H]-phosphatidylinositol (4)-monophosphate, [^{3}H]-phosphatidylinositol (4,5)-bisphosphate (1–5 Ci/mmol).
4. 1.5 mL screw-top Sarstedt tubes.
5. Perspex radiation safety shield.
6. HPLC system equipped with Partisphere strong anion exchange (SAX) columns (Whatman) and connected to either an on-line β-radiodetector or a fraction collector. If a fraction collector is used, it will be necessary to quantitate fractions using a β-scintillation counter.

2.2. Measurement of PI 3-Kinase Activity In Vitro

1. The following solutions can be prepared from analytical grade reagents dissolved in autoclaved deionized water. The solutions are stored at 4°C and are stable for up to 3 mo:
   a. Lysis buffer: e.g., 1% [v/v] Nonidet P-40, 100 m*M* NaCl, 10 m*M* iodoacetamide, 10 m*M* NaF, 20 m*M* Tris, pH7.4. Prior to use, the buffer is supplemented with the protease inhibitors PMSF (1 m*M*), leupeptin (1 μg/mL), pepstatin (1 μg/mL), and antipain (1 μg/mL), as well as the phosphatase sodium orthovanadate (1 m*M*).
   b. Phosphate-buffered saline (PBS).
   c. Washing buffer: 0.5 *M* LiCl, 100 m*M* Tris-HCl, pH 7.4.
   d. Lipid kinase assay buffer: 5 m*M* MgCl, 0.25 EDTA, 20 m*M* HEPES, pH 7.4.
   e. [γ-^{32}P]-ATP (3000 Ci/mmol).
2. 1 *M* HCl.
3. Chloroform:methanol (1:1 v/v).
4. Protein A- and protein G-Sepharose.
5. Phosphatidylinositol (PtdIns) and phosphatidylserine (PtdSer) in solid form; phosphatidylinositol (4)-monophosphate and phosphatidylinostiol (4,5)bisphosphate (1 mg/mL in chloroform stored for up to 6 mo at –20°C).

6. 1.5 mL screw-top Sarstedt tubes.
7. Probe sonicator.
8. Silica gel 60 TLC plates (19 cm × 19 cm) sprayed with 1% potassium oxalate and air dried.
9. TLC tank containing 1-propanol: 2 *M* acetic acid (65 : 35 v/v).
10. Whatman 3MM paper cut to size of TLC tank.
11. Iodine.
12. XAR-5 film (Kodak), exposure cassettes, X-ray film developer.

3. Methods

3.1. Measurement of D-3 Phosphoinositide Lipids

1. Cells must be depleted of phosphate prior to labeling with [^{32}P]Pi by washing three times in 50 mL phosphate-free balanced salts solution. Between washes, cells are incubated at 37°C for 10 min (*see* **Note 1**).
2. After phosphate depletion, cells are resuspended in balanced salt solution supplemented with 5% dialyzed FCS and 20 m*M* HEPES. After addition of [^{32}P]Pi, cells are incubated at 37°C for appropriate times (*see* **Note 2**).
3. After incubation at 37°C, the cells are washed three times in 50 mL of phosphate-free balanced salt solution to remove unincorporated [^{32}P]Pi. Cells are then resuspendended in Dulbecco Modified Eagle Medium (DMEM) at required cell concentration in balanced salt solution at approx 2 × 10^7 cell/mL.
4. Stimulate 120 μL aliquots at 37°C in 1.5-mL screw-top Sarstedt tubes.
5. Terminate incubations by addition of 500 μL chloroform /methanol/H_2O (32.6/65.3/2.1.% v/v) to produce a homogenous primary extraction phase (*see* **Note 3**).
6. Separate phases by addition of 200 μL chloroform containing 10 μg/mL Folsch lipids and 200 μL 2.4 *M* HCl, 5 m*M* tetrabutylammonium sulphate. Vortex and centrifuge (1000*g*, 5 min) to separate phases.
7. Remove lower phase (approx 800 μL) carefully into fresh 1.5-mL Sarstedt tube already containing 400 μL of 0.1 *M* HCl, 5 m*M* EDTA. Vortex and centrifuge (1000g, 5 min) to separate phases (*see* **Note 4**).
8. Remove lower phase into clean Sarstedt tubes and dry *in vacuo* (*see* **Note 5**).
9. Once dried, the extracted lipids must be deacylated by the addition of 1 mL 25% (w/v) methylamine/methanol/N-butanol (4 : 4 : 1) followed by incubation at 53°C water bath for 40 min. Samples are then cooled rapidly on ice for 5 min and dried *in vacuo* (*see* **Note 5**).
10. Add 0.5 mL H_2O followed by 0.6 mL N-butanol/petroleum ether (bp 40–60°C)/ethyl formate (20/4/1 v/v) to the dried deacylated lipids. Vortex and centrifuge (1000*g*, 5 min). Carefully remove the upper organic phase and discard. Wash the lower, water soluble phase with a further 0.6 mL of N-butanol/petroleum ether (bp 40–60°C)/ethyl formate mix. Vortex, centrifuge and discard upper phase as above. Dry lower phase *in vacuo*.
11. Redissolve pellet in 100 μL H_2O (*see* **Note 6**), and analyze deacylated [^{32}P]-labeled lipids by HPLC using a 12.5-cm Whatman Partisphere SAX column.

Samples are eluted from the column using a gradient based on buffers A (water) and buffer B (1.25 *M* $(NH_4)_2HPO_4$] (adjusted to pH 3.8 with H_3PO_4 at 25°C) at a flow rate of 1 mL/min: 0 min, 0% B; 5 min, 0% B; 45 min, 12% B; 60 min, 30% B; 61 min, 100% B; 65 min, 100% B; 66 min, 0% B; 90 min, 0% A and B.

12. Eluates can be analyzed with an on-line radiodetector or can be collected in fractions (0.5 mL) and quantitated by β-scintillation counting. Eluted peaks are compared to retention times for standards prepared from commercial [^{3}H] phosphoinositides and [^{32}P]-labeled D-3-phosphoinositides prepared as described in **Subheading 3.2.** An example of a typical HPLC separation of the water soluble deacylation products from [^{32}P]Pi- labeled leukaemic cell line is shown in **Fig. 2**.

3.2. Measurement of PI 3-Kinase Activity In Vitro

1. Immunoprecipitates should be prepared using either protein A/G-Sepharose depending on Ig subclass of antibody employed (*see* **Note 7**).
2. Wash immunoprecipitates three times in lysis buffer containing nonionic detergent such as Nonidet P-40 (*see* **Note 8**), twice in buffer without detergent, twice in phosphate buffered saline, twice in 0.5*M* LiCl, 100 m*M* Tris, pH 7.2, once in water and once in lipid kinase assay buffer. Resuspend immunoprecipitates in 40 μL of lipid kinase buffer and place at room temperature (*see* **Note 8**).
3. Make a mixture of 1 mg/mL PtdIns and 1 mg/mL PtdSer and disperse by sonication in 1 m*M* EDTA, 25 m*M* HEPES, pH 7.4 (*see* **Note 9**).
4. Add 50 μL of the sonicated PtdIns/PtdSer mixture to the immunoprecipitates. Initiate reaction by adding a mixture of 10 μCi [γ-^{32}P]-ATP and 100 μ*M* ATP in a vol of 10 μL.
5. Incubate at room temperature, agitating every minute or so. It is advisable to perform time course experiments to investigate linearity of the reaction, but 15–20 min incubation periods are usually sufficient.
6. Stop the reaction by adding 100 μL 1*M* HCl. To extract the lipids, add 200 μL of chloroform/methanol (1 : 1), vortex 20 s and centrifuge for 5 min to separate phases.
7. Recover and wash the lower chloroform phase by placing in a new tube containing 400 μL methanol/1 *M* HCl (1 : 1 v/v). Vortex and centrifuge to separate phases (1000*g*, 5 min).
8. Transfer the lower chloroform phase to a new tube and dry *in vacuo*.
9. Resuspend dried lipids in 50 μL chloroform and spot onto a dry TLC plate previously impregnated with 1% potassium oxalate (*see* **Note 10**). Spot 5–10 μL drops at a time and dry each drop using a low-power hair dryer. It is also advisable to spot on 10 μL of standard PtdIns(4)P and PtdIns(4,5)P_2 solutions as standards.
10. Place TLC plate into TLC tank containing1-propanol: 2 *M* acetic acid (65 : 35 v/v) and Whatman 3MM paper which has been saturated with the running solvent (*see* **Note 10**).
11. Once the solvent front has reached the top, remove the TLC plate, air-dry and place in TLC tank containing iodine to stain the substrate PtdIns and standard PtdIns(4)P and PtdInd(4,5)P_2. Remove from iodine tank and expose to X-ray

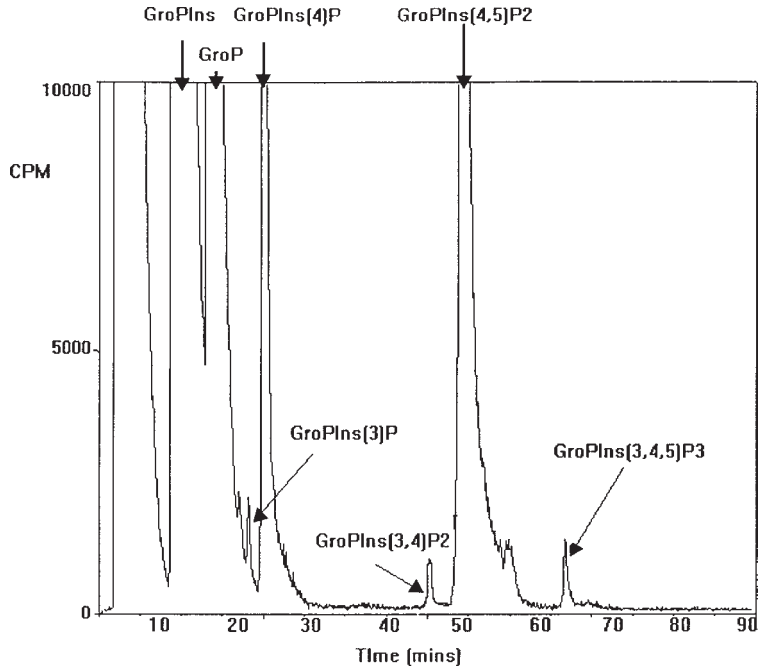

Fig. 2. HPLC elution profile of deacylated [^{32}P]-labeled phosphoinositides derived from CD28-activated Jurkat cells.

film at –70°C to identify phosphoinositide lipids (*see* **Note 11**). An example of a typical TLC separation of the D-3 phosphoinositide lipid products of an in vitro PI 3-kinase assay is shown in **Fig. 3**.

4. Notes

1. Balanced salt solution is cell-dependent but for leukaemic T-cell lines such as Jurkat cells, phosphate-free DMEM (Gibco) is appropriate.
2. Concentrations of cells and [^{32}P]Pi required for the assay are cell-dependent but 2×10^7 and 1 mCi/mL, respectively, is suitable for labeling of Jurkat cells. Incubation times for metabolic labeling are also cell dependent and may vary from 60 min to several hours.
3. Samples may be stored at –20°C at this stage.
4. Generally, the lower phase can be removed using a 200 μL gel loading tip which is immersed through both the upper and lower phases so that the tip rests on the bottom of the tube. The lower phase can then be carefully removed from the bottom upwards, exerting care to avoid taking any upper phase.
5. Once the [^{32}P]-labeled lipids have been separated into chloroform, they should not be stored but rather they should be dried *in vacuo* immediately followed by deacylation. Similarly, after deacylation, the cooled lipid extracts should be dried

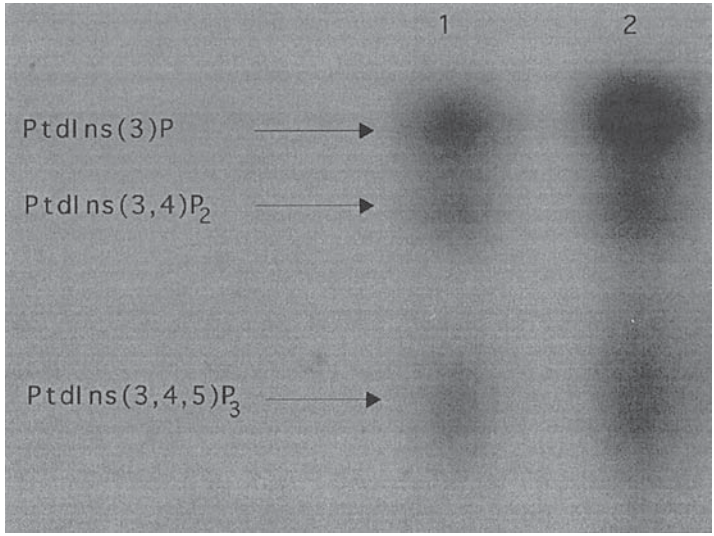

Fig. 3. TLC separation of D-3 phosphoinositide lipids derived from in vitro lipid kinase assay of p85 immunoprecipitates using PtdIns as a substrate. Immunoprecipitates are derived from resting (lane 1) or RANTES-stimulated (lane 2) human T lymphocytes.

immediately *in vacuo*. This drying step can be performed overnight and the deacylated samples can then be stored at –20°C if necessary prior to washing with N-butanol/petroleum ether (bp 40–60°C)/ethyl formate (20/4/1 v/v).

6. Redissolved [^{32}P]-labeled phosphoinositide lipid solutions should be centrifuged (1000*g*, 2 min) and/or filtered (0.45 μm filter) to remove any debris prior to HPLC separation on a Partisphere SAX column.
7. Antibody can be directed against a specific PI 3-kinase subunit (e.g., p85 α/β, p110 α/β/δ, etc.), or either surface receptors (PDGF-R, the T-cell costimulatory molecule CD28) and cellular proteins (e.g., *Src* tyrosine kinases), which may coassociate with PI 3-kinase(s). Alternatively, the assays may be performed on any peptide fragments (suitably immobilized on beads) that have been used to precipitate cellular proteins.
8. Many lipid kinases are sensitive to detergents ***(9,10)***. Do not use sodium dodecyl sulphate (SDS) in lysis buffers since SDS kills lipid kinase activity. Moreover, Nonidet P-40 inhibits PI 3-kinase activity but potentiates PI 4-kinase activity. It is therefore crucial to remove nonionic detergents such as Nonidet P-40 and to remove all detergent from the immunoprecipitates by thoroughly washing them in 1 mL volumes of the indicated solutions prior to assaying them for associated lipid kinase activity. Immunoprecipitates are generally washed by pelleting protein A/G beads by centrifugation (1000*g*, 3 min), aspirating 90% of the supernatant and adding 1 mL of the next washing solution. During the last washing step,

after aspirating 90% of the supernatant, the remaining 10% of the supernatant should be removed using a flat-end Hamilton syringe, with minimal disruption of the protein Sepharose A/G beads. It is critical to keep all washing buffers on ice and to perform all centrifugation steps at 4°C to minimise risk of protease and phosphatase activity.

9. Sonication should be performed on ice with a probe sonicator (usually during the immunoprecipitation step). Typically, three 10 s bursts interspersed by periods of 30 s is sufficient. However, the precise sonication time will depend on the power of the particular sonicator and whether it is in tune. The PtdIns appears cloudy at first and should eventually clear. Sonicating too long results in oxidized, precipitated lipid. Do not store the substrate mixture, but rather make up sufficient amounts of PtdIns/PtdSer mixture for the assay and discard any excess. The sonicated mixture of PtdIns and PtdSer may be further diluted as required, to obtain optimal substrate concentration.
10. TLC plates can be impregnated with 1% potassium oxalate by simple immersion or by spraying. The 1-propanol : 2 *M* acetic acid running solvent for TLC, allows good separation of PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, although PtdIns(3)P and PtdIns(3,4)P_2 are not resolved too well from PtdIns(4)P, PtdIns(4,5)P_2 respectively. This may be a problem when immunoprecipitated proteins coassociate with other lipid kinases such as PtdIns 4-kinase. In such circumstances, it may be appropriate to use the following TLC separation system to separate PtdIns(3)P from PtdIns(4)P ***(11)***. Silica gel 60 plates are immersed face up for 10 s with gentle stirring in trans-1,2-diamino-cyclohexane-N,N,N',N'-tetraacetic acid (CDTA) solution. This solution is prepared by stirring a mixture of 0.9 g CDTA in 30 mL 0.33 *M* NaOH followed by the addition of 60 mL ethanol. After immersion in CDTA solution, the plates are air-dried and then activated for 10 min at 110°C. The TLC developing solution is prepared by stirring together 35 mL methanol, 32 mL chloroform, and 24 mL pyridine in a bottle in a fume hood (to avoid exposure to harmful pyridine). Add 6.3 g boric acid and shake until dissolved. Add 4 mL water and 1.6 mL concentrated formic acid and transfer solution to the TLC tank. Saturate tank using Whatman 3MM paper attached to the tank walls. Plate should be air-dried 2–3 h prior to autoradiography. It is important to note that iodine detection of the separated lipids is impossible due mainly to pyridine on the TLC plate.
11. Identity of phosphoinositide lipids can be verified in four ways:
    a. Comparison with known cold standards that are identified by iodine staining;
    b. Comparison with radiolabeled standards that are identified by autoradiography);
    c. HPLC analysis of the radiolabeled phosphoinositide lipids after the lipids have been recovered from the plate by scraping and subsequently deacylated (*see* **Subheading 3.1., step 9**);
    d. Sensitivity of in vitro lipid kinase activity to PI 3-kinase inhibitors such as wortmannin ***(12)*** and LY294002 ***(13)*** or reagents such as adenosine (inhibits PI 4-kinase but does not affect PI 3-kinase) ***(10)*** and Nonidet P-40 (inhibits PI 3-kinase, but potentiates PI 4-kinase activity) ***(9,10)***. These inhibitors and

reagents can be added to the lipid kinase assay mixture at appropriate concentrations (*see* **Subheading 3.2., step 4**).

References

1. Vanhaesebroeck, B., Leevers, S., Panayatou, G., and Waterfield, M. D. (1997) Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem. Sci.* **22,** 267–271.
2. Toker, A. and Cantley, L. (1997) Signalling through the lipid products of phosphoinositide 3-OH kinase. *Nature* **398,** 673–678.
3. Jackson, T., Stephens, L., and Hawkins, P. T. (1992) Receptor specificity of growth factor-stimulated synthesis of 3-phosphorylated inositol lipids in Swiss 3T3 cells. *J. Biol. Chem.* **267,** 16,627–16,636.
4. Stephens, L., Jackson, T., and Hawkins, P. T. (1993) Synthesis of phosphatidylinositol 3,4,5-trisphosphate in permeabilised neutrophils regulated by receptors and G proteins. *J. Biol. Chem.* **268,** 17,162–17,172.
5. Stephens, L., Hawkins, P. T., and Downes, C. P. (1989) Metabolic and structural evidence for the existence of a third species of polyphosphoinositide in cells: D-phosphatidyl-*myo*-inositol 3-phosphate. *Biochem J.* **259,** 267–276.
6. Whitman, M., Downes, C. P., Keeler, M., Keller, T., and Cantley, L. (1988) Type 1 phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol 3-phosphate. *Nature* **33,** 644–646.
7. Varticovski, L., Druker, B., Morrison, D., Cantley L., and Roberts, T. (1989). The colony stimulating factor-1 receptor associates with and activates phosphatidylinositol 3-kinase. *Nature* **342,** 699–701.
8. Morgan, S. J., Smith, A. D., and Parker, P. J. (1990) Purification and characterisation of bovine brain type I phosphatidylinositol kinase. *Eur. J. Biochem.* **191,** 761–767.
9. Carpenter, C. L. and Cantley, L. C. (1990) Phosphoinositide 3-kinases. *Biochemistry* **29,** 11,147–11,156.
10. Ward, S. G., Reif, K., Ley, S., Fry, M. J., Waterfield, M. D., and Cantrell, D. A. (1992) Regulation of phosphoinositide kinases in T cells: evidence that phosphatidylinositol 3-kinase is not a substrate for T cell antigen receptor-regulated tyrosine kinases. *J. Biol. Chem.* **267,** 23,862–23,869.
11. Walsh, J. P., Caldwell, K. K., and Majerus, P. W. (1991) Formation of phosphatidylinositol 3-phosphate by isomerisation from phosphatidylinositol 4-phosphate. *Proc. Natl. Acad. Sci. USA* **88,** 9184–9187.
12. Ui, M., Okada, T., Hazeki, K., and Hazeki, D. (1995) Wortmannin is a unique probe for an intracellular signalling protein phosphoinositide 3-kinase. *Trends Biochem. Sci.* **20,** 303–307.
13. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1995) A specific inhibitor of phosphatidylinositol 3-kinase 2-(4-morpholinyl)-8-phenyl-4H-one-benzopyran-4-one (LY294002). *J. Biol. Chem.* **269,** 5241–5248.

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Glycosaminoglycan Binding Assays

Arlene J. Hoogewerf and Gabriele S. V. Kuschert

1. Introduction

The interaction of a select group of chemokines with glycosaminoglycans has been demonstrated previously, both by affinity coelectrophoresis ***(1)*** and by cell-surface binding assays and immobilized binding heparin assays ***(2)***. The significance of the interaction of chemokines with glycosaminoglycans has been demonstrated for a limited subset of chemokines. A first example is the interaction of heparin with Platelet Factor 4, resulting in the neutralization of the anticoagulant property of heparin ***(3)***. Secondly, using both immobilized heparin and cell-surface proteoglycans, it has also been demonstrated that chemokines oligomerize on proteoglycans. This oligomerization on solid surfaces can cause an increase in the local concentration of chemokine, which in turn affects the interaction of the chemokine with adjacent high affinity cell-surface receptors ***(2)***. More recently it has been demonstrated that the cell-surface proteoglycans on T cells play a role in the regulation of the anti-HIV-I activity of RANTES. Removal of cell-surface heparin sulfate, but not chondroitin sulfate from a human T-cell line, rendered the cells resistant to the antiviral effects of RANTES ***(4)***. These authors demonstrate the importance of understanding the interaction of different chemokines with different proteoglycan types, since a chemokine may not bind all proteoglycans equally.

This chapter describes an assay for examining the interaction of chemokines with proteoglycans. This solid phase binding assay, using immobilized heparin, radiolabeled chemokines, and various competitor proteoglycans, is simple to perform and allows the analysis of multiple samples in a short time period. Competition of the binding of a radiolabeled chemokine to immobilized heparin by either unlabeled ligand or competitor glycosaminoglycans yields 50%

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inhibition constants (IC_{50} values). For a given chemokine, comparison of the IC_{50} values obtained with various competitor molecules allows one to determine the relative potency and selectivity of the competitor molecules.

2. Materials

2.1. Reagents

1. Heparin-Sepharose (Pharmacia #17-0467-01); alternatively Sigma #H5263 for heparin-acrylic beads; Sigma #H6508 for heparin-agarose; Sigma #H0402 for heparin-agarose with terminal aldehyde-coupling.
2. Sepharose (unsubstituted; Pharmacia).
3. 96-well filter plates (Millipore [Bedford, MA] Multiscreen MADVN6510).
4. Soluble glycosaminoglycan competitors: Heparin–Sigma H3393; Chondroitin sulfate–Sigma C8529; Dermatan Sulfate–C2413; Heparan Sulfate–H5393.
5. Unlabeled chemokines (Peprotech, R & D Systems) or other proteins.
6. [^{125}I]-chemokines—Amersham (specific activity 2000 Ci/mmol).
7. Buffer Reagents: Bovine serum albumin (Sigma), HEPES buffer (Gibco-BRL), $MgCl_2$, $CaCl_2$, NaCl (reagent grade).

2.2. Equipment

1. Vacuum filtration apparatus for separating free and bound radioligand in 96-well filtration plates (Millipore MultiScreen System or equivalent).
2. Microbeta Scintillation Counter for measuring radioactivity in 96-well plates (Wallac, Turku, Finland, or other manufacturer).
3. Platform shaker to accommodate 96-well plates.

3. Methods

1. Rehydrate a defined amount of heparin-sepharose, wash according to the manufacturer's suggestions, and remove the fines. Resuspend the wet gel in Binding Buffer (1 m*M* $CaCl_2$, 5 m*M* $MgCl_2$, 0.5% BSA, 50 m*M* HEPES, pH 7.2) at a stock concentration of 0.25 mL wet gel/ mL total volume (for heparin-sepharose from Pharmacia, 1 g dry weight ≈ 4 mL wet gel, and the concentration of heparin is 2 mg/mL of wet gel, giving a concentration of heparin of 0.5 mg/mL). In this text, all concentrations of the immobilized heparin refer to the mass of the heparin, rather than the mass of dry gel or volume of wet gel.
2. Dilute the heparin-sepharose stock solution to the appropriate concentration to deliver 0.015 μg/well to 1.5 μg/well immobilized heparin in a vol of 50 μL Add heparin-sepharose to the wells (50 μL/well) of a Millipore filtration plate.
3. Add competitor (other proteins, competitive ligands, or glycosaminoglycans) to the filtration plate in a vol of 30 μL, preparing duplicate to quadruplicate wells for each point. For the glycosaminoglycan competitors used in these studies, the concentration of glycosaminoglycans are 0–2 mg/mL, spanning 4 orders of magnitude with 10–12 points.

4. Add a constant amount of radioiodinated ligand in a vol of 20 μL. For the studies described in this chapter, we are using 5 n*M* unlabeled MCP-1 (monocyte chemoattractant protein-1), with a tracer concentration of 0.25–0.5 n*M* [^{125}I]MCP-1.
5. Incubate the plates for 30 min—8 h at room temperature with shaking.
6. Separate free and bound radioiodinated ligand by vacuum filtration.
7. Wash the heparin-sepharose with 3 × 200 μL wash buffer (0.15–0.5 *M* NaCl in Binding Buffer).
8. Allow the plates to dry for 30 min at room temperature.
9. Add 30 μL scintillation fluid to each well.
10. Measure the radioactivity with a calibrated Microbeta Scintillation Counter.
11. Analyze the data using GraFit Software ***(5)*** or other appropriate curve-fitting software to calculate the 50% inhibition concentration of the competitor. We use an equation, based on a single site model: $B/Bmax^{app} = 1/(1 + [L]/IC_{50})$), where B = cpm bound, $Bmax^{app}$ = cpm bound in the absence of competing ligand, [L] = competing ligand, and the IC_{50} = [radioligand] + K_d ***(6)***.

4. Notes

The study of the binding of radiolabeled proteins with immobilized glycosaminoglycans will depend on several factors, including the length of incubation, the concentration of both the ligand and the immobilized glycosaminoglycan, the temperature, and the buffer composition, including ionic strength. All of these factors can influence the measurement of the relative affinity between chemokines and glycosaminoglycans and should be carefully defined in order to obtain meaningful and reproducible data.

In setting up the conditions for performing the binding studies, it is advisable to use two to four concentrations of both the immobilized heparin and radiolabeled ligand and perform a time-course of the binding reaction. Two important pieces of data can be obtained from this initial experiment: First, one can examine the approach to equilibrium and choose the appropriate experimental time frame. Second, using a range of concentrations of both the immobilized heparin and radiolabeled ligand in a "checkerboard analysis," one can determine a concentration of both variables that achieves an acceptable signal-to-noise ratio but avoids ligand depletion. Ligand depletion occurs when a significant amount (greater than 15–20%) of the total ligand added to the system is bound at equilibrium. This causes a significant reduction in the concentration of free ligand and results in complicated association kinetics which shift the equilibrium curve to the right. For a more comprehensive review of receptor-ligand interactions and ligand depletion kinetics, the reader is referred to the volume edited by E. C. Hulme ***(7)***.

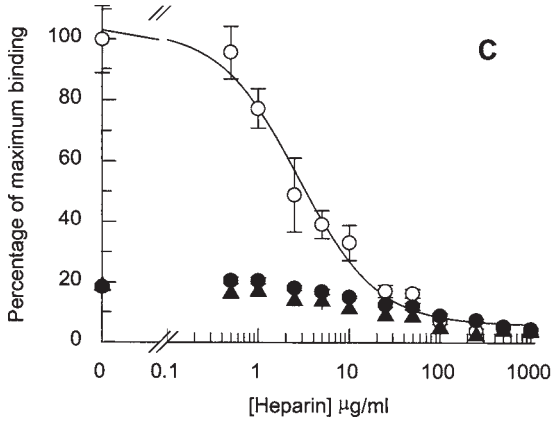

Fig. 1. Chemokines have different relative affinities for heparin in an immobilized-heparin competitive binding assay. Radiolabeled chemokines were incubated with either Heparin-Sepharose beads (○), Sepharose beads (▲), or without beads (●) in the presence of increasing concentrations of heparin. Competition assays with MCP-1 were performed as described in the Methods section. Each point represents the mean ± S.E.M. of triplicate determinations, and the experiment shown is a representative of 2–4 experiments for each chemokine. The maximal binding was 3000–5000 cpm, which corresponded to 15–20% of the total added radioactivity.

After the initial experimental conditions have been chosen with respect to kinetics and concentrations of ligands and immobilized heparin, variations in buffer composition, such as magnesium ion concentration, pH, and total ionic strength, can be examined. Finally, a measurement of the nonspecific binding of the system should also be made.

Figure 1 shows the binding of the immobilized heparin and the competition by soluble heparin. The data represented by the open circles represents the binding of [^{125}I]MCP-1 to heparin-Sepharose beads, and the competition of this binding by soluble heparin, resulting in an IC_{50} value of 3.3 ± 0.4 (µg/mL for heparin. In this experiment the nonspecific binding was assessed by repeating the competition binding experiment using unsubstituted Sepharose beads (closed triangles) or without beads (closed circles) rather than Heparin-Sepharose beads (open circles). This experiment compares the binding of the radiolabeled MCP-1 to the binding of other components of the system, including the Sepharose beads or the filter plates. The low level of binding (5–20% of the maximum) in the presence of the unsubstituted sepharose beads and without beads clearly demonstrates that the MCP-1 interacts with heparin to a much greater extent than the beads or filter plates.

References

1. Witt, D. P. and Lander, A. D. (1994) Differential binding of chemokines to glycosaminoglycan subpopulations. *Curr. Biol.* **4,** 394–400.
2. Hoogewerf, A. J., Kuschert, G. S. V., Proudfoot, A. E. I., Borlat, F., Clark-Lewis, I., Power, C. A., and Wells, T. N. C. (1997) Glycosaminoglycans mediate cell surface oligomerization of chemokines. *Biochemistry* **36,** 13,570–13,578.
3. Lane, D. A., Denton, J., Flynn, A. M., Thunberg, L., and Lindahl, U. (1984) Anticoagulant activities of heparin oligosaccharides and their neutralization by platelet factor 4. *Biochem. J.* **218,** 725–732.
4. Oravecz, T., Pall, M., Wang, J., Roderiquez, G., Ditto, M., and Norcross, M. A. (1997) Regulation of anti-HIV-1 activity of RANTES by heparan sulfate proteoglycans. *J. Immunol.* **159,** 4587–4592.
5. Leatherbarrow, R. J. (1992) GraFit Version 3.01, Erithicus Software Ltd., Staines, UK.
6. Cheng, Y. and Prusoff, W. H. (1973) Relationship between the inhibition constant [K_I] and the concentration of an inhibitor that causes a 50% inhibition [I_{50}] of an enzymic reaction. *Biochem. Pharmacol.* **22,** 3099–3108.
7. Hulme, E. C. and Birdsall, N. J. M. (1992) Strategy and tactics in receptor-binding studies, in *Receptor-Ligand Interactions* (Hulme, E. C., ed.), Oxford University Press, Oxford and New York, pp. 63–176.

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CFU-A Assay for Measurement of the Antiproliferative Effects of Chemokines on Murine Early Hemopoietic Progenitors

Gerard J. Graham and Mary G. Freshney

1. Introduction

One of the more intriguing features of the hemopoietic system is the ability of a single phenotypically homogeneous stem cell to give rise—through differentiation and development—to all the phenotypically diverse mature immunohemopoietic cell types ***(1)***. The stem cell therefore sits at the heart of the hemopoietic system, and it is at this cellular level that the entire complex system can ultimately be regulated. For this reason, an understanding of the nature of the factors acting to regulate processes such as stem cell proliferation is central to our overall ability to understand the functioning of the hemopoietic system ***(2)***.

Careful biological analyses of cells occupying the primitive end of the hemopoietic system have demonstrated that there is not a single hemopoietic stem cell but that there is a complex, hierarchical, overlapping population of cells, all of which fulfill the basic criteria required of a stem cell, i.e., self-renewal and differentiation capacity ***(1)***. For this reason we now talk of a stem-cell compartment comprising these overlapping cell types, rather than of a stem cell per se. Bioassays ***(3)*** for the most primitive members of the hemopoietic stem cell compartment are very complex, expensive, and time-consuming, and thus most analysis of stem cell regulatory molecules is carried out on more easily assayable but slightly more mature stem cells. Such assays are typified by the in vivo murine CFU-S assay ***(4)***. The CFU-S assay is still a cumbersome and expensive assay and does not lend itself easily to the role of screening

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assay for use during biochemical purification of candidate stem cell regulators. We have developed a simple and inexpensive in vitro assay which we believe is detecting a cell that is phenotypically indistinguishable from that detected by the murine CFU-S assay, and have used this assay, the CFU-A assay ***(5,6)***, to purify to homogeneity, a murine, macrophage derived inhibitor of hemopoietic stem cell proliferation ***(7)***. Sequencing revealed this molecule to be identical to the chemokine MIP–1α which we have now generated in recombinant form, and have demonstrated its ability to inhibit stem cell proliferation both in vitro and in vivo ***(8,9)***. Many of our subsequent studies with this stem cell regulatory chemokine have relied heavily on the robust but facile CFU-A assay, which we describe in **Subheading 3.1.**

Essentially this assay relies on the ability of synergizing cytokines, Stem Cell Factor (SCF), Macrophage Colony Stimulating Factor (M-CSF), and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) to trigger the proliferation and differentiation of CFU-S-like hemopoietic stem cells in soft agar. The assay is set up using two layers of agar. The underlayer consists of a 0.6% agar/medium mixture which contains the growth factors and cytokines. The top layer is 0.3% agar/medium and contains the cells. Following 11 d of culture, macroscopic colonies develop (> 2mm) which are clonal in origin and which routinely contain macrophages and granulocytes. The cells giving rise to these colonies have previously been shown to share many characteristics with the murine CFU-S stem cells and thus we believe this assay to be an in vitro correlate of the in vivo CFU-S assay ***(6)***. As well as allowing the quantitation of stem cell numbers in murine bone marrow or peripheral blood, this assay can also be used to measure the cell cycle status of the murine stem cells. This is done, as described in **Subheading 3.3.** by using the cell cycle specific cytotoxic drug, cytosine-arabinofuranoside in conjunction with the routine CFU-A assay. In this way effects of chemokines on cell cycle status can be measured. An easier measure of the stem cell inhibitory properties of chemokines can be obtained using the "direct addition assay" ***(10)*** in which chemokines are added directly to the developing CFU-A assay plates and the inhibitory effects of the chemokine registered as inhibition of macroscopic colony formation.

2. Materials

2.1. The CFU-A Assay

2.1.1. Preparation of Agar Solutions

1. Pipet 100mL sterile ultra-pure water into a sterile conical flask with a foil lid and add 1.2 g of agar.
2. Dissolve by boiling for 2 min.
3. Transfer to a water bath at 55°C.

2.1.2. Preparation of Alpha Stock

1. The components of the alpha stock are as follows: Alpha medium (10-L powder pack); MEM Vitamin stock (100X); Gentamicin sulphate (200 mg); Ultra-pure water.
2. Stir the water and powder for about 1 h on a heated stirrer to dissolve all the ingredients properly. Do not heat above 37°C. Add MEM Vitamin stock and Gentamicin and make to 3 L with ultra-pure water.
3. Always use glassware or plastic that is dedicated for tissue culture use only.
4. Prefilter before final filtration through a sterile 0.22 μm filter (*see* **Note 1**).
5. Aliquot and store at –20°C.

It is useful to have premeasured aliquots of 21 mL stored, so that rapid thawing of the alphastock is possible when making up the final medium.

2.1.3. Preparation of $\alpha \times 2$ Medium

This prepares 50 mL of medium, scale up as required: 21 mL Alpha stock; 25 mL Donor horse serum; 1 mL Glutamine; 3 mL, $NaHCO_3$. Mix and equilibrate to 37°C in a sterile bottle.

2.1.4. Preparation of Growth Factors

An inexpensive way of obtaining growth factors is to generate them in-house by preparing a conditioned medium harvested from the appropriate growth factor expressing cell lines. For the CFU-A assay, the L929 mouse lung fibroblast cell line (ATCC CCl1), is a valuable source of M-CSF (CSF-1), and the AF1-19T rat fibroblast cell line transformed with the malignant hystiocytosis sarcoma virus (MHSV) is a source of GM-CSF ***(11)***.

2.1.4.1. Preparation of Conditioned Medium

1. Cell lines: L929, AF1-19T. Tissue culture flasks and Roller bottles. Medium: Dulbecco's MEM/10% FCS (D/10).

   Conditioned medium is usually made in large vol in order to guarantee continuity over a period of time, i.e., 9 mo to 1 y.
2. Grow the cell lines in polystyrene flasks or roller bottles (850 cm^2) in medium with 10% FCS until they are 50% confluent.
3. Replace the medium with up to 300 mL of fresh medium and grow for a further 4 d, collect this medium and store at –20°C.
4. Replace this medium with fresh medium for 4 d.
5. Thaw the stored medium and combine the two lots.
6. If many cells have detached from the substrate, spin down the medium before prefiltering (*see* **Note 1**) and final sterile filtration through a 0.22 μm.

7. Aliquot and store at –20°C.
8. Test in the assay by titrating from 100–400 mL/6 cm dish with an active amount of the other conditioned medium.

If the conditioned medium is to be made serum-free, grow the cells to confluence in medium containing serum as in **Subheading 2.1.4.1.**, then change to serum free medium and incubate up to 7 d before harvesting the medium.

If it is thought to be an advantage to increase the activity of the conditioned medium, this may be achieved by concentrating it fourfold using a 10kD molecular weight cut-off membrane. This should be done before the final sterile filtration. There are many types and makes of ultra-filtration equipment available on the market.

2.1.4.2. Recombinant Growth Factors

If funds are available it is considerably more convenient to purchase purified recombinant growth factors available from suppliers such as R&D Systems Inc.

As substitutes for conditioned media, the recombinant factors should be used in the CFU-A assay at the following concentrations: 0.2 ng/mL r Murine GM-CSF; 6 ng/mL r Human CSF-1; 12 ng/mL SCF.

2.2. Staining the CFU-A Assay Plates

2.2.1. INT Staining

Make a stock of 1 mg/mL INT [*2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride*] in phosphate-buffered saline (PBS) or Hanks' BSS.

1. Dissolve in boiling water bath or heated stirrer.
2. Remove undissolved particles by filtration using Whatman No.1 filter paper.
3. Sterilize by filtration through a sterile 0.22 μm-filter and store in the dark at 4°C.

2.2.2. May-Grunwald and Giemsa Staining

1. Glutaraldehyde solution: Make a 2.5% solution of glutaraldehyde by diluting 25% stock solution 1/10 with PBS. This solution should be prepared fresh each time.
2. May-Grunwald and Giemsa Buffer: 17 mL 1.0 *M* Citric acid; 46 mL 1.0 *M* $Na_2HPO_4.2H_2O$. Adjust the pH to 5.6–6.0 and make up to 2 L with distilled water. Store at room temperature.

2.3. The CFU-A Cycling Assay

2.3.1. Preparation of Fischers/20% Donor Horse Serum (F/20)

Fischers medium is required for the stem cell suicide assay (*see* **Subheading 3.3.**) and is made by mixing the following: 437.5 mL Sterile ultra-pure

water; 50 mL Fischers 10X; 5 mL Glutamine; 7.5 mL $NaHCO_3$; 100 mL (20%) Donor Horse Serum (DHS).

2.3.2. Preparation of Ara-C solution

1. Weigh out Ara-C (*cytosine b-D-arabinofuranoside*) and dissolve in PBSA to give 1 mg/mL.
2. Filter-sterilize using a 0.22-μm syringe end filter.
3. Dilute 1/4 in Fischers/20% DHS.

Make this solution up fresh each time and keep on ice until you are ready to use it.

3. Methods

3.1. The Murine CFU-A Assay

3.1.1. Preparation of the Assay Under Layer

1. Add equal volumes of 1.2% agar at 55°C and 2X alpha medium at 37°C and allow to equilibrate to 37°C for at least 10 min. Use plastic disposable pipets for all manipulations involving agar (*see* **Note 2**).
2. Calculate the amount of under layer medium required to carry out the experiment and pipet this into a bottle. Add the growth factors to this (usually 10% of the vol of the under layer of each of them if the source is conditioned medium). Return the bottle of medium containing growth factors to the water bath at 37°C.

3.1.2. Preparation of Murine Bone Marrow Cells

1. 6–12-wk-old mice are killed either by gassing with CO_2 or cervical dislocation. Soak the fur with 70% alcohol. Lift the fur around the midline and make a small cut through the fur and skin. Using your fingers pull gently away from each hand and pull down the skin over the animals hind legs (*see* **Note 3**). Remove the femurs using sterile scissors and forceps by first cutting away as much tissue as possible above and below it and then cutting first below the knee joint and then into the hip joint.
2. Remove as much muscle and tissue as possible using swabs wetted with 70% alcohol and place the femurs in cold PBS.
3. Take the femurs to the tissue culture area, wipe them again with 70% alcohol, and place them in a sterile Petri dish containing 5 mL of Fischers medium with 20% DHS.
4. Using sterile scissors and forceps, pick up a femur and carefully cut off the knee (distal) end of it. Insert a 21-gage needle into the bone and cut the other (proximal) end off. Transfer to a universal container, containing 10 mL F/20 and suck medium in and out of the syringe and bone three times to flush out the cells from the marrow cavity (*see* **Note 4**). Resuspend the marrow core by sucking up and down several times using a 10-mL pipet.
5. Spin cell at 250*g* for 10 min and discard the supernatant.

6. Resuspend the cells in F/20 medium, 4 mL/femur and count the nucleated cells in a hemocytometer after staining with methylene blue, or count the cells on an electronic cell counter after first lysing the red cells with Zapoglobin. Each femur will yield 10–15 × 10^6cells.
7. Dilute the cells to 10^6/mL and keep on ice until you are ready to add them to the upper layer of agar/medium.

3.1.3. Preparation of the Assay Upper Layerin

1. Dissolve 0.6 g agar in 100 mL sterile ultra-pure water by boiling for 2 min as in **Subheading 2.1.1.**
2. Transfer to a 55°C water bath for at least 10 min.
3. Dispense the required volume of 2X alpha medium, equilibrated to 37°C, add an equal vol of 0.6% agar, and keep at 37°C until ready to use. Allow the medium/agar to cool to 37°C before adding the cells. **Important:** Do not rush this step or you will kill the cells if the medium is too hot.
4. Add bone marrow cells to give a concentration of 5 × 10^3/mL if you are using 3-cm dishes and 2.5 × 10^3/mL (10^4 cells/dish) if you are using 6-cm dishes. If the concentration of cells is 10^6/mL, this will be 5 μL or 10 μL of cell suspension/dish.
5. Dispense 1 mL or 4 mL into each dish as appropriate and allow to set for 15–20 min.
6. To cut down the risk of fungal and bacterial contamination, the Petri dishes should be contained in a plastic box. Clean the box and lid internally and externally with 70% alcohol before use (*see* **Note 5**).
7. Place in a humid incubator at 37°C with an atmosphere of 10% CO_2, 5% O_2 and air. If a low oxygen incubator is not available, gas cylinders containing the CO_2/O_2 mixture may be rented. In this case a perforated sheet of perspex can be laid on top of four silicone bungs (~3 cm long) and sterile water added to the box. Place the dishes on top of the tray and replace the lid, which should have a hole drilled in it. Seal the lid to the box with insulation tape and gas the box through the hole in the lid for 10 min. Seal the hole in the lid with tape and place the box at 37°C.

Count the colonies after 8 d to observe the maximum inhibitory effect. After 11 d the control CFU-A colonies are usually >2 mm in diameter but by 8 d the inhibited colonies will have a smaller, tighter appearance. The incidence of macroscopic colonies should be in the range of 100–200/10^5 plated cells depending on the strain of mouse that is used. For most accurate results use the 6-cm dishes, as described, with 10^4 cells. Three cm dishes are useful when material is scarce or valuable.

3.1.4. Analysis by Direct Addition of Chemokine activity

For simple and rapid analysis of inhibitory activity, the sample/fraction of inhibitor to be tested for activity is added directly to the culture dish for the

duration of the assay (*see* **Note 6**). For preliminary experiments, and if material is in short supply, 3-cm dishes should be used for these assays as measurement of activity is based on colony size. When precise protein concentrations are not available, it is normally permissible to add the inhibitory material in vol up to 10% of total assay vol (i.e., 200 μL for a 3-cm dish assay). When the material is active, the colonies generated in the assay will be smaller in diameter and look much more dense than the control colonies.

The colonies should be examined and counted at between days 8 and 10, with the precise time of scoring being dictated by the time at which colonies in the control plates reach a diameter of 2 mm. Leaving the assays more than 1 d beyond this time can often result in the inhibitory effect being lost owing to the overgrowth of the colonies in the inhibited plates. This direct addition assay has been used to measure the inhibitory activity of both MIP-1α and other stem-cell inhibitory molecules such as TGF-β ***(12,13)***.

Specifically, the direct addition assay is carried out as follows:

1. Lay out the appropriate number of labeled 3-cm (6-cm) Petri dishes on a clean tray.
2. Add the required vol of inhibitor to the dishes (*see* **Note 7**).
3. Add 1 mL (4 mL) of medium/agar mixture to each dish and immediately mix gently by swirling the dish three times.
4. Allow the under layers to set.
5. These may be stored at 4°C for up to 5 d prior to addition of the upper layer of agar.

3.2. Staining the CFU-Assay

3.2.1. INT Staining

To facilitate the counting of the colonies stain with a solution of INT 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride hydrate.

To stain CFU-A colonies:

1. Add 0.2 mL stain to 3-cm Petri dishes or 1 mL to 6-cm dishes.
2. Replace in the plastic box.
3. Return the dishes to the CO_2 incubator overnight.

3.2.2. Colony Counting

Count the colonies using a stereoscopic microscope with a zoom lens. The sizing is made easier if a grid is used to measure the size. This is simply made by photocopying graph paper on to an acetate sheet and placing this below the dish to be counted. As the colonies are of macroscopic size it is possible to count them with the naked eye but if several researchers are collaborating and

comparing results it is more objective if they standardize the measurement and counting of colonies by using microscope and grid.

3.2.3. Preparation and Staining of Agar Films

According to the requirements of the assay, it is often desirable not to stain the assay plates with INT, but to make "agar films" of the colony containing upper agar layer. It is possible to fix the agar films with gluteraldehyde and to stain them using May-Grunwald and Giemsa following transfer to glass slides. This procedure allows colonies to be examined *in situ* and their cell content identified using morphological criteria.

3.2.3.1. Glutaraldehyde Fixation of Agar Films

1. Add 2.5% glutaraldehyde solution to the Petri dish, (1 mL to 3-cm dishes, 5 mL to 6-cm dishes) and leave for 10 min. **Note:** All manipulations involving gluteraldehyde should be carried out in a fume hood.
2. Add 20 mL of distilled water to 9-cm Petri dishes. The Petri dishes should be labeled so that when the agar films are added to them, it is possible to identify each film at the end of the fixation process.
3. Run the end of a small spatula round the circumference of the top agar film.
4. Slide the top agar film only into the Petri dish of water. (Do not worry if the underlayer slips in too, it can be separated later.)
5. Replace the water 3–4 times during the next 48 h to destain, i.e., to remove the phenol red and excess glutaraldehyde. Do this using a 25-mL pipet, and taking care not to suck up the agar film.
6. Prepare a large sandwich box of water, 5–10 cm deep, and sit this on top of a piece of black paper.
7. Label clean glass slides of a size that will cover the diameter of the agar films.
8. Cut squares of Whatman chromatography paper (DE81) which will also cover the agar film.
9. Float the agar films out of the Petri dishes one at a time and into the sandwich box.
10. Place the corresponding glass slide below the film and carefully lift it out of the water on to the slide, you will need to use the fingers of your other hand to steady the agar and keep it from sliding off again!
11. Wet a piece of filter paper and place on top of the film on the slide.
12. Leave on a tray to dry overnight.
13. Remove the filter paper from the slide by running warm water over it.
14. Rub your finger gently over the surface of the film to remove fibers.
15. Place the slides in a slide rack ready for staining.

3.2.3.2. Staining of Agar Films

1. Prepare May-Grunwald (MG) stain fresh each time used; 71 mL up to 500 mL with buffer (cytoplasmic stain).
2. Prepare Giemsa stain fresh; 6.6 mL up to 500 mL with buffer.

3. Place the slides in a staining dish and add the MG stain.
4. Incubate at 37°C for 20 min.
5. Remove MG stain by running in water to replace the stain, drain the slides.
6. Add the Giemsa stain and incubate for 40 min at 37°C.
7. Run water into the dish to replace the stain.
8. Rinse with buffer.
9. Drain, air-dry, and mount with a coverslip.

The CFU-A colonies derived from murine bone marrow are composed mainly of macrophages (90–95%) when conditioned media are used as sources of growth factors. Using recombinant growth factors (rGFs) increases the percentage of neutrophils in the colonies with a resultant decrease in macrophages. This appears to be linked to the amount of CSF-1 in the conditioned medium or the concentration of rCSF-1 used.

3.3. Analysis of Stem-Cell Proliferative Status Using the CFU-A Suicide Assay

Cell suicide is a method of assessing the proportion of cells engaged in DNA synthesis where direct measurement of DNA synthesis is precluded. It depends upon the fact that certain cytotoxic agents which are lethal specifically for cells in S-phase, such as [^{3}H] thymidine, hydroxyurea, or cytosine arabinofuranoside (Ara-C), kill only those cells which are synthesizing DNA. With appropriate controls, a reduction in the proportion of colonies due to the cytotoxic agent becomes a measure of the proportion of colony-forming cells synthesizing DNA and thus transiting S-phase during the time when they were exposed to the cytotoxic agent.

One problem with such assays when performed on hemopoietic stem cells is the very low rate of cycling of stem cells. It is estimated that at any point in time only between 5 and 10% of hemopoietic stem cells are in active cell cycle. Measurement of the effects of proliferative inhibitors therefore becomes difficult against this low cycling background. To alleviate this problem, mice are pretreated with one of a range of myelosuppressive chemicals prior to bone marrow harvesting. The intention here is to use myelosuppressive agents that will induce the stem cells into cell cycle as part of the normal processes of regeneration following insult. Typically as many as 50% of bone marrow stem cells obtained at appropriate times after such treatments will be in active cell cycle thus making the measurement of inhibition of proliferation much easier.

3.3.1. 5-Fluorouracil (5FU) Induction of Stem Cell Cycling in Mice

1. Weigh out 5FU and add to PBS to give 15 mg/mL.
2. Mix gently at 37°C by rotating for 30 min to dissolve. Keep dark.

3. Inject mice via the tail vein with 150 mg/kg, i.e., 3 mg/20 g mouse. Inoculation vol is 0.2 mL/20 g mouse.

3.3.2. The Stem-Cell Cycling Assay

1. Incubate marrow from mice that have been treated with 5FU for 2 d (*see* **Subheading 3.3.1.**) in paired 15 mL centrifuge tubes, containing 2–5 × 10^6 cells in 1 mL of F/20 in the presence of inhibitor or control solution for 4 h.
2. Add 0.1 mL of a 250 mg /mL solution of Ara-C to one tube and an equal volume of medium to the other.
3. Gas the tubes with 5% CO_2/air and incubate for 1 h at 37°C.
4. Spin the cells at 250*g* for 10 min and remove the medium.
5. Wash the cells three times to remove the Ara-C and resuspend in 1 mL medium.
6. Count the cells and dilute to 10^6/mL in preparation for addition to the culture dishes. Seed 10^5 cells in each 6 cm dish.
7. For accurate statistics ten, 6 cm dishes should be set up for each point, ± Ara-C.
8. Count the colonies after 11 d incubation in the conditions as described in **Subheading 3.1.**

The proliferative status of the stem cells in this assay is determined by applying the following formula:

$$\text{Mean of no. of colonies per + Ara-C plates/Mean of no. of colonies per – Ara-C plates} \times 100\%$$

4. Notes

1. Prefiltration is usually necessary before final sterile filtration to remove any particulate material which would block and therefore shorten the life of the sterile filter.
2. Agar will block the tip of a glass pipet unless it is discarded into hot disinfectant.
3. Always check the size of the animals' spleen. An enlarged spleen would suggest that the animal has an infection and therefore should not be used.
4. Although there is a sufficient number of cells in one femur to set up the assay, it is usual to take the femurs from at least two mice and mix the cells.
5. As well as wiping the box with 70% EtOH at the start of the experiment it is advisable to wipe the outside of the box with 70% EtOH whenever it is removed from the incubator to withdraw and examine dishes before the experiment is terminated. This will eliminate the possibility of spores entering the box when the lid is removed.
6. Crude or purified preparations of chemokines may bind to polystyrene, therefore all storage and dilution tubes should be of polypropylene. When adding to the dishes, add to no more than three dishes at a time, followed immediately by the agar/medium mixture.
7. This is usually around 100 ng/mL but in the first instance it is usual to make a titration from 0–200 ng/mL. e.g., 0, 25, 50, 100, 150, 200 ng/mL.

References

1. Graham, G. J. and Wright, E. G. (1997) Haemopoietic stem cells. *Int. J. Exp. Pathol.* **78,** 197–218.
2. Graham, G. J. (1997) Growth inhibitors in haemopoiesis and leukaemogenesis, in *Molecular Haemopoiesis* (Whetton, A. D., ed.), *Baillieres Clinical Haematology*. **10,** 539–559.
3. Wright, E. G. and Pragnell, I. B. (1992) The stem cell compartment: Assays and negative regulators. *Current Topics Microbiol. Immunol.* **177,** 137–149.
4. Till, J. E. and McCulloch, E. A. (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiation Res.* **14,** 213–222.
5. Pragnell, I. B., Wright, E. G., Lorimore, S. A., Adam, J., Rosendaal, M., Delamarter, J. F., Freshney, M., Eckmann, L., Sproul, A., and Wilkie, N. (1988) The effect of stem cell proliferation regulators demonstrated with an *in vitro* assay. *Blood* **72,** 196–201.
6. Lorimore, S. A., Pragnell, I. B., Eckmann, L., and Wright, E. G. (1990) Synergistic interactions allow colony formation *in vitro* by murine haemopoietic stem cells. *Leuk. Res.* **14,** 481–489.
7. Graham, G. J., Wright, E. G., Hewick, R., Wolpe, S. D., Wilkie, N. M., Donaldson, D., et al. (1990) Identification and characterisation of an inhibitor of haemopoietic stem cell proliferation. *Nature* **344,** 442–444.
8. Dunlop, D. J., Wright, E. G., Lorimore, S., Graham, G. J., Holyoake, T., Kerr, D. J., et al. (1992) Demonstration of stem cell inhibition and myeloprotective effects of SCI/rhMIP-1α *in vivo*. *Blood* **79,** 2221–2225.
9. Graham, G. J., Mackenzie, J., Lowe, S., Tsang, M L-S., Weatherbee, J. A., Issasson, A., et al. (1994) Aggregation of the chemokine MIP-1α is a dynamic and reversible phenomenon: Biochemical and biological analyses. *J. Biol. Chem.* **269,** 4974–4978.
10. Graham, G. J., Freshney, M. G., Donaldson, D., and Pragnell, I. B. (1992) Purification and biochemical characterisation of human and murine stem cell inhibitors (SCI). *Growth Factors* **7,** 151–160.
11. Franz, T., Lohler, J., Fusco, A., Pragnell, I. B., Nobis, P., Padua, R., et al. (1985) Transformation of mononuclear phagocytes *in vivo* and malignant histiocytosis caused by a novel murine spleen focus-forming virus. *Nature* **315,** 149–151.
12. Maltman, J., Pragnell, I. B., and Graham, G. J. (1993) Transforming growth factor β: Is it a downregulator of stem cell inhibition by macrophage inflammatory protein 1a? *J. Exp. Med.* **178,** 925–932.
13. Maltman, J., Pragnell, I. B., and Graham, G. J. (1996) Specificity and reciprocity in the interactions between transforming growth factor beta and macrophage inflammatory protein-1 alpha. *J. Immunol.* **156,** 1566–1571.

23

Downmodulation and Recycling of Chemokine Receptors

Matthias Mack and Detlef Schlöndorff

1. Introduction

Binding of chemokines to their receptors induces a variety of biological responses, ranging from early and rapid events such as receptor phosphorylation and calcium influx to prolonged responses such as cellular migration ***(1,2)***. Downmodulation of chemokine receptors from the cell surface becomes detectable after a few minutes and reaches its maximum after 20–30 min. Recycling of the receptor after removal of the ligands is a slow process, that requires 15–120 min ***(3,4)***. As chemokine receptors function as coreceptors for HIV-type 1 and 2, the amount of receptors present on the cell surface during and after incubation with various ligands substantially influences the likelihood of HIV infection ***(3,5)***. Assays to measure downmodulation and recycling of chemokine receptors can therefore help to determine the potential of ligands to reduce cellular HIV infection. Different ligands can show marked differences in their spectum of functional responses on chemokine receptors (**Figs. 1** and **2**). An interesting example is the effect of RANTES, Met-RANTES, and AOP-RANTES ***(6)*** on hCCR5. In contrast to RANTES and Met-RANTES, AOP-RANTES almost completely downmodulates CCR5 from the cell surface, although it induces less calcium influx and even blocks RANTES-induced cell migration ***(3)***. Moreover, AOP-RANTES, in contrast to RANTES, prevents recycling of CCR5 after wash-out of chemokines as shown in **Fig. 3**.

Chemokine receptor expression on the cell surface can be quantified by flow cytometry (FACS). This requires antibodies for receptor detection and cells that express the appropriate receptor. Antibodies can be either monoclonal antibodies (MAbs) against extracellular portions of the receptor, polyclonal

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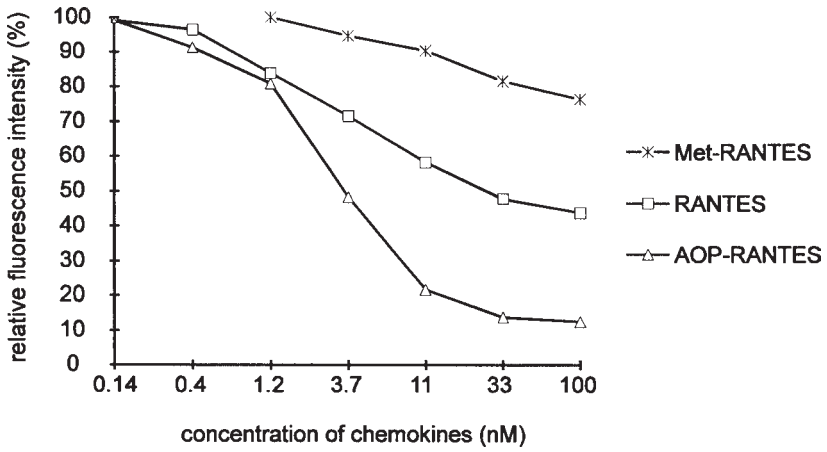

Fig. 1. Downmodulation of CCR5 from the surface of stably transfected CHO-CCR5 cells with RANTES, AOP-RANTES, and Met-RANTES. Staining of CCR5 was performed with the CCR5-antibody MC-1. (Adapted with permission from **ref. *3***.)

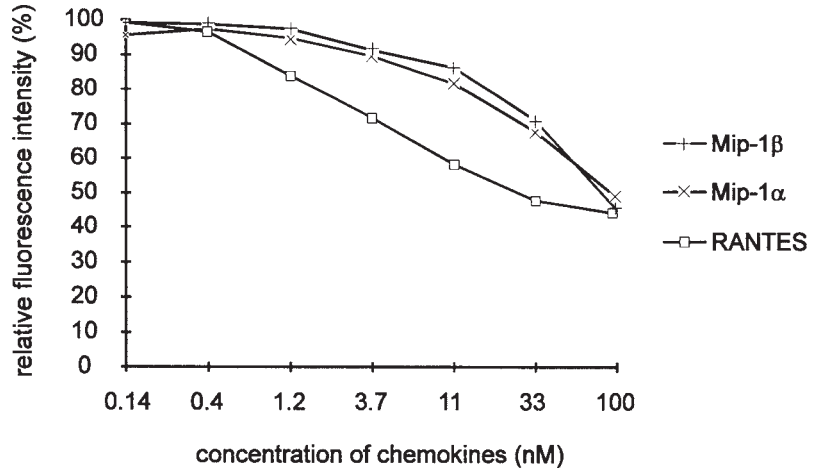

Fig. 2. Downmodulation of CCR5 from the surface of stably transfected CHO-CCR5 cells with RANTES, Mip-1α and Mip-β as shown in legend. Staining of CCR5 was performed with the CCR5-antibody MC-1. (Adapted with permission from **ref. *3***.)

antibodies (PAbs) generated against receptor peptides, or antibodies against N-terminal tags (e.g., FLAG, HA) of genetically engineered receptors. Antibodies against tags and in many cases polyclonal antipeptide antibodies are unable to detect chemokine receptor expression on primary cells. These reagents however, are in general suitable for stably transfected overexpressing

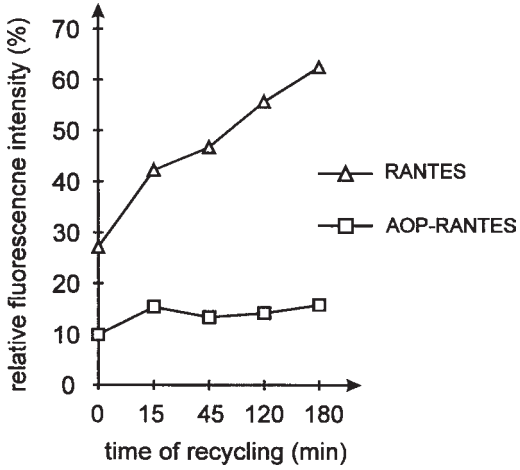

Fig. 3. Recycling of the CCR5 after downmodulation with RANTES (30 n*M*) or AOP-RANTES (30 n*M*). The assay was performed with stably transfected CHO-CCR5 cells.

cell lines. When using stable transfection, care has to be taken to choose the right cell line, as some cells lack the appropriate machinery for downmodulation of the receptor. In addition, only those antibodies are suitable that show no altered binding to the receptor after previous exposure to chemokines. As downmodulation of chemokine receptors is only seen at 37°C, experiments performed in parallel at 37°C and on ice can distinguish between downmodulation of the receptor and reduced binding of the antibodies.

2. Materials

1. Cell lines or primary cells expressing chemokine receptors.
2. Appropriate serological reagents for detection of chemokine receptors.
3. Chemokines.
4. 96-well plates (U-bottom).
5. ELISA shaker.
6. Multichannel pipet.
7. Incubator at 37°C.
8. Cooled centrifuge for 96-well plates.
9. Flow cytometer.

3. Methods

3.1. Downmodulation Assay

1. In parallel, preload the wells of two 96-well U-bottom plates with chemokines at various concentrations in a vol of 100 µL. Chemokines are diluted in the same

culture medium, that is used for the cells. As the control, preload two wells per lane with culture medium only.
2. Preincubate one plate at 37°C in an incubator and the other plate on ice.
3. Gently remove cells from culture flasks and add 2×10^5 cells/well in a vol of 50 μL culture medium. The final concentration of the chemokines in each well will then drop to 66.6%. Before adding the cells to the plate on ice also precool the cells.
4. Incubate the two plates for 20–30 min at 37°C and on ice, respectively. Shake the plates every 10 min on an ELISA shaker at 1000 rpm. Avoid spillage of medium from one well to the other.
5. Centrifuge the plates at 400*g* for 1 min at 2°C and decant the medium by briefly inverting the plates. Immediately cover the plates with a paper towel to avoid cross-contamination. Suspend the cells from the pellet by briefly vortexing the plates on an ELISA shaker at 1000 rpm.
6. Place both plates on ice and add precooled receptor antibodies to all wells, which contain chemokines and one of the two wells with medium. The latter serves as 100% control, whereas the other well with medium is incubated with an isotype control antibody and serves as 0% control.
7. If you have to use secondary antibodies, perform all further steps including washing and centrifugation at 2°C, to prevent recycling of the receptor.
8. Quantitate the fluorescence signal by FACS analysis and calculate the relative fluorescence intensity as follows: (mean channel fluorescence [exp.] – mean channel fluorescence [0% control]) / (mean channel fluorescence [100% control] – mean channel fluorescence [0% control]).

3.2. Recycling Assay

1. Perform a downmodulation assay as described in **Subheading 3.1., steps 1–4** with two modifications: Use only one plate, which is incubated at 37°C, and add 1×10^6 cells/well.
2. After downmodulation transfer 30 μL of each well to a new 96-well plate on ice. These data points correspond to the 0 min recycling value.
3. Wash out the chemokines by four washing steps with medium (200 μL each) at RT as described in **Subheading 3.1., step 5**.
4. Add 160 μL of medium and further incubate at 37°C. Take out aliquots of 40 μL at four different time points up to 2 h and also transfer them to the new 96-well plate on ice.
5. Simultaneously stain and analyze all cells as described in **Subheading 3.1., steps 5–8**, and calculate the relative fluorescence intensity for each time point with the appropriate controls.

4. Notes

1. When working with adherent cells, remove them gently from the culture flask with PBS containing 1.5 m*M* EDTA. Do not use trypsin solutions.

2. For shaking and centrifugation of 96-well plates, cover them with adhesive foil.
3. To get rid of the supernatant after centrifugation of the 96-well plates, quickly invert the plate once and immediately cover it with a paper towel. The remaining fluid in general is below 10 μL per well and no cells are lost.
4. Downmodulation and recycling does not take place on ice to a significant amount. Never warm the cells to room temperature during washing or centrifugation.
5. For FACS analysis of receptor expression, a gate should be set on the living cells in the forward and sideward scatter dot plots.

References

1. Baggiolini, M., Denwald, B., and Moser, B. (1997) Human chemokines: an update. *Annu. Rev. Immunol.* **15,** 675–705.
2. Aramori, I., Zhang, J., Ferguson, S. S. G., Bieniasz, P. D., Cullen, B. R., and Caron, M. G. (1997) Molecular mechanism od desensitization of the chemokine receptor CCR5: receptor signaling and internalization are dissociable from its role as an HIV-1 co-receptor. *EMBO J.* **16,** 4606–4616.
3. Mack, M., Luckow, B., Nelson, P. J., Cihak, J., Simmons, G., Clapham, P. R., Signoret, N., Marsh, M., Stangassinger, M., Borlat, F., Wells, T. N. C., Schlöndorff, D., and Proudfoot, A. E. I. (1998) AOP-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. *J. Exp. Med.* **187,** 1215–1224.
4. Solari, R., Offord, R. E., Remy, S., Aubry, J.-P., Wells, T. N. C., Withehorn, E., Oung, T., and Proudfoot, A. E. I. (1997) Receptor-mediated endocytosis of CC-chemokines. *J. Biol. Chem.* **272,** 9617–9620.
5. Amara, A., Gall, S. L., Schwartz, O., Salamero, J., Montes, M., Loetscher, P., Baggiolini, M., Virelizier, J.-L., and Arenzana-Seisdedos, F. (1997) HIV coreceptor downregulation as antiviral principle: SDF-1a-dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. *J. Exp. Med.* **186,** 139–146.
6. Simmons, G., Clapham, P. R., Picard, L., Offord, R. E., Rosenkilde, M. M., Schwartz, T. W., Buser, R., Wells, T. N. C., and Proudfoot, A. E. I. (1997) Potent inhibition of HIV-1 infectivity in makrophages and lymphocytes by a novel CCR5 antagonist. *Science* **276,** 276–279.

24

Analysis of Chemokine Receptor Endocytosis and Recycling

Nathalie Signoret and Mark Marsh

1. Introduction

Endocytosis regulates the cell-surface expression of many seven transmembrane G-protein coupled receptors (7TM-GPCRs) and has been implicated in both desensitization following agonist-induced activation and resensitization ***(1,2)***. The internalization mechanism for many 7TM-GPCRs has not been clearly established. However, a clear paradigm is emerging for the β_2-adrenergic receptor (β_2AR) ***(1,2)***. For β_2AR, agonist binding induces activation of heterotrimeric G proteins, which in turn activate one or more members of the family of G-protein coupled receptor kinases (GRKs). GRK-mediated phosphorylation of the receptor enhances the binding of β-arrestins, proteins that uncouple heterotrimeric G protein activation and link the receptor to clathrin-coated pits by binding to clathrin heavy chains ***(1–3)***. Coated vesicles internalize the receptors and deliver them to endosomes, where they can be dephosphorylated and recycled to the cell surface ***(4)***.

This mechanism also appears to operate for chemokine receptors. For CCR1, CCR5, CXCR2, and CXCR4, there is evidence that chemokines induce receptor endocytosis ***(5–8)***. For CXCR4, endocytosis can also be induced by phorbol esters ***(5,6)***. The phorbol ester-induced CXCR4 endocytosis occurs through clathrin-coated pits ***(6)***. Clathrin-coated vesicles have also been implicated in the uptake of CCR5: overexpression of GRKs and β-arrestins can enhance ligand-induced CCR5 internalization ***(9)***, and internalization can be inhibited by treatments known to perturb clathrin-coated vesicle-mediated endocytosis ***(10)***. Agonist and—for CCR5 at least ***(10)***—antagonist-induced

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endocytosis of CCR5 and CXCR4 results in delivery of the receptors to endosomes, from which they can recycle to the cell surface ***(5,6)***.

A variety of methods have been developed to study cell-surface receptor internalization. A number of these methods have now been adapted for chemokine receptors. The choice of assay depends primarily on the question to be addressed and the reagents available. To follow receptors from the cell surface to intracellular sites biochemically, radiolabeled receptor-specific antibodies or chemokines have been used. However the chemokines, especially the CC chemokines, frequently bind to several different receptors as well as cell surface proteoglycans. We have preferred to use receptor-specific monoclonal antibodies. Where these antibodies are unavailable and receptors have been cloned, N-terminal epitope tags have proved an effective way to follow the receptor. In most cases, the peptide epitope (YPYDVPDYA) from influenza virus hemagglutinin (HA) has been used in conjunction with a commercially available monoclonal antibody (MAb) 12CA5 (Boehringer Mannheim UK Ltd., Lewes, East Sussex, UK). However, the affinity of this antibody for N-terminally tagged receptors is not high and other epitopes may be more effective. When using antibodies on live cells, it is important to know whether the antibodies themselves influence internalization (by mimicking ligand or cross-linking receptors), whether antibody binding is inhibited by an agonist or antagonist, or whether the antibody itself inhibits agonist or antagonist binding. In some cases, antibody Fab fragments can be used as alternative probes. However, it is essential that the affinity is such that receptor-ligand complexes are internalized into cells together and that there is no significant dissociation of cell-surface receptor-ligand complexes.

Receptor-specific antibodies can also be used to analyze chemokine receptor endocytosis morphologically. Antibodies can be bound to cell-surface receptors and the distribution of these antibodies determined after ligand or, for CXCR4, phorbol ester-induced endocytosis, the primary antibodies being detected by second layer fluorescent-conjugates (immunofluorescence) or colloidal gold conjugates (immuno-electron microscopy). Alternatively the distribution of receptor can be monitored on fixed cells by conventional immunofluorescence staining. Fluorescent chemokines have also been used to examine chemokine receptor internalization. However, the problems of binding specificity discussed above can also compromise the use of these reagents. An alternative means to study cloned receptors is to use the green fluorescent protein (GFP). Studies with β_2AR and CCR5 suggest that addition of GFP to the COOH-terminus of the receptor does not perturb ligand-induced receptor internalization ***(5,11)***. However, care should be taken to ensure the same is true for other 7TM-GPCR-GFP constructs. The intracellular distribution of receptor can be examined by double- or triple-label immunofluorescence

microscopy using well-characterized organelle-specific antibodies. However, even confocal microscopy cannot identify unequivocally receptor-containing intracellular compartments and it is important to complement light microscopy with appropriate electron microscopy. Alternatively, subcellular distributions can be examined using cell fractionation.

Whether following cell-surface receptors into cells or looking at the distribution of receptors following chemokine or phorbol ester-induced endocytosis, it is essential to have the means to distinguish the cell-surface pool of receptor from the intracellular pool. We usually use brief washes in low pH media to remove cell-surface antibodies and ligands. Thus, cells treated with low pH medium are compared to cells maintained in normal medium. If the elution medium pH does not fall below pH 3.0, the cells can usually be returned to culture without any apparent deleterious effects, allowing receptor recycling to be analyzed. If the cells do not require reculture more aggressive low pH washes (~pH 2.0) can be used. It is often the case that a low pH wash does not destroy antibody-binding epitopes on the receptor. Thus ligand can be eluted from the cell surface and the receptor numbers then quantitated by antibody binding.

Here we provide the basic methods we use to study chemokine-receptor trafficking. These methods have been used in conjunction with chemokines and chemokine antagonists, and with other agents (e.g., phorbol esters) that can induce receptor endocytosis. In the following protocols, we refer only to the use of chemokines. We use the operational terms endocytosis and recycling to avoid alternative expressions such as down-modulation or down-regulation, desensitization, and resensitization, which may be used to reflect the functional state of the receptor or cell.

2. Materials

2.1. Radiolabeled Antibodies

See **Subheading 3.1.** for the preparation of radiolabeled antibodies.

2.2. Buffers and Solutions

All solutions are prepared in deionized H_2O. Chemicals are from Sigma (Poole, UK) unless indicated otherwise.

1. Binding medium (BM): To prepare 100 mL, add 10 mL of 10X RPMI-1640 without bicarbonate (Gibco Ltd., Paisley, Scotland), 1 mL of 1 *M* HEPES, pH 7.0, and 1 mL of 20% BSA solution to 80 mL of H_2O. Adjust the pH to 7.3–7.4 with 5 *M* sodium hydroxide and bring to 100 mL with H_2O. BM at 37°C and 4°C are used in experiments. **Note:** If binding reactions are done outside a buffered CO_2 environment, as is usual for fixed cells or 4°C incubations, the omission of bicarbonate helps maintain the medium pH.

2. Acid-strip medium (EM): To prepare 100 mL of EM, add 10 mL of 10X RPMI-1640 without bicarbonate, 1 mL of 1 *M* MES, and 1 mL of 20% BSA solution to 80 mL of water. Adjust the pH to 2.0–3.0 as needed using conc. HCl and bring to 100 mL with H_2O. Keep at 4°C.
3. Phosphate-buffered saline (PBS) containing Ca^{++} and Mg^{++}. Keep at 4°C.
4. Cell-harvesting solution: 0.2 *M* NaOH is used to harvest adherent cells at the end of experiments.
5. PFA fixative: 3% para-formaldehyde (PFA) in PBS should be prepared in advance and kept in aliquots at –20°C or prepared fresh. PFA should be prepared in a fume hood.
6. Quench solution: 50 m*M* NH_4Cl in PBS to quench free aldehyde groups after fixation.
7. BSA cushion: 5% BSA in PBS is prepared in advance and stored at –20°C.
8. PBS containing 0.2% fish-skin gelatin (PBS-gelatin). For permeabilized cells (*see* **Subheading 3.**), 0.05% Saponin is added (PBS-gelatin-Sap).
9. PBS containing 0.05% Saponin (PBS-Sap).
10. Cell supports:
    a. Clean, sterile 13-mm diameter glass coverslips.
    b. Poly-D-Lysine coated glass coverslips. Clean 13-mm diameter coverslips are covered with a drop of PBS containing 1 mg/mL poly-D-lysine, and left for 15 min at room temperature. The coverslips are washed in deionized H_2O, dried, and stored in a dry Petri dish.
11. Mountant for fluorescence microscopy can be obtained from several commercial sources, but we make our own. 2.4 g Moviol (Calbiochem) are added to 6 g analytical grade glycerol and 6 mL H_20 in a 50-mL Falcon tube at room temperature. The dissolution of the Molviol is completed by the addition of 12 mL 0.2 *M* Tris-HCl, pH 8.5, and the solution is incubated at 50°C for 10 min. The solution is clarified if necesssary by centrifugation at 5000*g* for 15 min. The solution is stored at –20°C in suitable aliquots.

2.3. Labeling Medium (LM)

Radiolabeled antibody is diluted in BM to an appropriate concentration. Preliminary experiments should be done to determine the binding kinetics and antibody avidity. Routinely we use antibodies at the K_d concentration to give approx half B_{max} epitope occupation.

2.4. Equipment

1. 37°C incubator (without CO_2 if BM is to be used) and/or 37°C water bath.
2. Horizontal reciprocal shaker (orbital shakers are less effective).
3. Gamma counter and γ counter tubes (75 × 12 mm, 5-mL tubes) (Sarstedt, Leicester, UK).
4. Refrigerated centrifuge; unless otherwise indicated centrifugations are at 200*g* for 5 min at 4°C.

5. Fluorescence microscope equipped with appropriate filter sets for detection of one or more fluorochromes and image capture system. If cells that grow flat are to be used, a conventional epifluorescence microscope is adequate. For thicker cells, a microscope equipped with a confocal laser scanning system is an advantage.

3. Methods

3.1. Radiolabeled Antibodies

We use ^{125}I-Bolton and Hunter reagent (Amersham International plc, Little Chalfont, UK) to radiolabel purified monoclonal antibodies (MAbs). This procedure routinely produces high specific activity reagents without significant loss of binding affinity or specificity. Bolton and Hunter reagent (0.5 mCi at about 2000 Ci/mmol, i.e., about 250 pmol) is dried onto the side of a 1.5-mL microfuge tube. Antibody (250 pmol) in approx 50 µL of 0.1 *M* borate buffer, pH 8.5, is added and the tube incubated for 20 min at room temperature with brief vortex mixing every 2 min. The reaction is stopped by addition of 0.2 *M* glycine in borate buffer, and the iodinated protein separated from reagents by gel filtration over an Econo-pac 10 DG column (Bio-Rad) eluted with PBS containing 0.25% gelatin and 0.02% sodium azide. Specific activities are determined by trichloroacetic acid (TCA) precipitation of aliquots taken before gel filtration, and are typically 300–600 Ci/mmol. Radioiodinated proteins are stored in small aliquots at −20°C and are stable for several months.

3.2. Method 1: Measuring Cell-Surface Receptor Levels

Cell-surface receptor levels can be measured directly using iodinated receptor-specific MAbs. If cells are treated with chemokines or other agents, and receptor internalization induced, antibody binding to treated cells will indicate the level of receptor down-modulation. **Note:** This is not a direct measure of endocytosis, but reflects the combined effects of endocytosis and recycling. Some steps in this protocol vary according to whether adherent cells or suspension cells are used.

3.2.1. Cells

1. Suspension cells: Cells from cultures in linear growth phase are collected, washed with fresh culture medium, and counted. The cell numbers required will depend to some extent on the level of receptor expression. For the T-cell line SupT1 for example, we use approx 6×10^6 cells/time point. Cells are collected by centrifugation, washed twice in cold BM, and resuspended in 1 mL BM/6×10^6 cells. One 1 mL aliquot of cells is transferred to a tube containing 5 mL of cold BM and held on ice as a T = 0 sample.
2. Adherent cells: Cells are seeded in 16 mm wells in 4- or 24-well tissue-culture plates (Nunc; supplied through Gibco) and grown for 2 d to a final density of

$1–2 \times 10^5$ cells/well. Usually, each experiment is performed in triplicate (i.e., 3 wells for each time point) and for kinetic analyses, 6 time points (0, 2, 5, 10, 30, 60 min) are used. The cells for separate time points are plated on separate 4- or 24-well plates. At the start of the experiment, the culture medium is removed by aspiration and cells are washed twice in cold BM. The 0 min plate is placed on ice and washed twice with 1 mL of 4°C BM.

3.2.2. Chemokine Treatment

3.2.2.1. Incubation

Cells are incubated in pre-warmed 37°C BM, in the presence or absence of chemokines. The concentration of agent used is established by titration. As a guide, we usually test chemokines in the 10 n*M*–10 μ*M* range for endocytosis activity. Suspension cells can be incubated in a single tube from which aliquots are removed. Care should be taken to ensure cells do not settle, especially during the longer incubations. However, cells should not be shaken too vigorously. At the required times, the incubations are stopped by rapidly transferring cells to 4°C as follows.

1. Suspension cells: At each time point, a 1-mL aliquot (6×10^6 cells) is transferred to a tube containing 5 mL of 4°C BM. All tubes are kept on ice until the last aliquots are collected. Then all cells are washed twice with 5 mL 4°C BM by centrifugation (*see* **Subheading 2.4.**).
2. Adherent cells: Plates are placed on ice and each well rapidly washed with 4°C BM. The plates are kept on ice until the final time point, then all cells are washed twice with 4°C BM by aspiration and addition of fresh 4°C BM.

Cells should be kept at 4°C for subsequent steps unless indicated otherwise.

3.2.2.2. Elution of Cell Surface-Bound Chemokines

If the antibody binding is inhibited by receptor-bound chemokine, the chemokine can be removed by briefly washing the cells in acidified medium (EM). Essentially the same protocol is used to remove surface-bound antibodies (*see* **Subheading 3.3.**).

1. Suspension cells: Cells are centrifuged, the BM removed by aspiration, and the cells gently resuspended in 5 mL 4°C EM for 3 min. The cells are then centrifuged, the EM removed, and the cells given 2 washes in 4°C BM. Note that cells should not be pipeted or vortexed but resuspended by inversion.
2. Adherent cells: The medium is removed by aspiration and replaced with EM. The cells are rinsed twice with 1 mL EM and the final rinse left on the cells for 3 min. The EM is aspirated away, replaced with BM, and the cells rinsed 4 times with 1 mL BM.

3.2.2.3. Antibody Labeling of Cell-Surface Receptors

1. Suspension cells: We find that labeling is more reliable if cells are fixed before incubation with antibody. Cells are fixed in 1 mL 3% PFA for 10 min, centrifuged, washed in 10 mL PBS, and incubated in 1 mL quench solution for 20 min at room temperature. Cells are then centrifuged again, washed in BM, and incubated in 1 mL LM/6 × 10^6 cells for 2 h at room temperature. The cells are centrifuged and resuspended into 3 mL BM. To separate the bound from remaining free radiolabeled antibody, 3 × 1 mL 5% BSA cushions are placed in γ counter tubes for each time point, and 1-mL aliquots of cell suspension gently layered onto the BSA. The tubes are centrifuged, the supernatant and cushion aspirated away, and the ^{125}I activity counted. (*See* **Note 1** for data normalization.)
2. Adherent cells: Cells are incubated with 300 µL LM/well for 2–4 h at 4°C on a reciprocal shaker set at approx 30 oscillations/min. Subsequently, the LM is aspirated away and the cells washed at least 4 times with 1 µL/well 4°C BM and twice in 4°C PBS. The PBS is removed by aspiration and 400 µL 0.2 *M* NaOH is added to each well. The lysed cells in NaOH are transferred to γ counter tubes. Each well is rinsed with 400 µL H_2O, the rinse added to the lysate, and the ^{125}I activity counted. (*See* **Note 1** for data normalization.)

3.3. Method 2: Measuring Receptor Endocytosis Using Iodinated Receptor-Specific Antibodies

Method 1 can be adapted to measure the rates and extents of receptor endocytosis directly ***(12)***. We have found this method useful for measuring phorbol ester-induced CXCR4 endocytosis where ligand-binding is not required ***(6)***. The method is not appropriate if antibody and chemokine bind to related sites on a receptor. This problem may be solved by using N-terminally epitope-tagged receptors expressed in appropriate cells and epitope-tag-specific antibodies that should not influence chemokine binding and signaling.

3.3.1. Cells

Set up as in **Subheading 3.2.**

3.3.2. Antibody Labeling of Cell-Surface Receptors

1. Suspension cells: Cells can be labeled in one tube using approx 1 mL 4°C LM/6 × 10^6 cells. (Suitable cell numbers are needed to determine both total cell-associated and acid-resistant radioactivity.) The cells are washed with 3 changes of 4°C BM by centrifugation.
2. Adherent cells: At least 4 wells of cells are needed for each time point and/or condition (2 duplicate wells for total cell counts and 2 duplicate wells for acid elution). The cells are labeled with LM at 4°C, as for Method 1.

3.3.3. Receptor Endocytosis

1. Suspension cells: Two aliquots of cells are removed and kept on ice as zero time points. The remaining cells are centrifuged and the cell pellet resuspended in

pre-warmed 37°C BM, or 37°C BM containing chemokine or phorbol ester, and incubated at 37°C. At the required times, aliquots of cells are transferred to tubes containing 5 mL 4°C BM and held on ice. At the end of the incubation, the cells are washed. Half of the cells for each time point are washed with EM as above to elute cell-surface antibody. Subsequently, all cells are washed in BM and the ^{125}I activity determined as for Method 1. Fixation is not required for this procedure.

2. Adherent cells: After washing away unbound label with several changes of 4°C BM, the cells are synchronously warmed to 37°C by replacing the cold BM with 1 mL 37°C BM, or 37°C BM containing chemokine or phorbol ester. The cells are then placed in a 37°C incubator. At the required times, the warm BM is removed to a 1.5 mL tube on ice (*see* **Note 2**) and 4°C BM added to each well. Half of the wells for each time point are treated with EM as described in **Subheading 3.2.2.2.** to remove labeled antibody molecules accessible on the cell surface (*see* **Note 3**). Following acid elution, all cells are collected, and the ^{125}I activity determined as for Method 1.

3.3.4. Endocytosis Rates

Endocytosis rates are determined by calculating the ratio of radioactivity in acid-treated cells as a proportion of total cell-associated activity (non-acid-treated cells).

3.4. Method 3: Detecting Internalized Chemokine Receptors by Fluorescence Microscopy

Receptor-specific antibodies can be used to analyze morphologically the cellular distribution of chemokine receptors, following ligand treatment. If the anti-receptor antibodies do not interfere with chemokine binding the protocols can be adapted to follow endocytosis and recycling of cell surface receptors (*see* **Notes 4** and **5**).

Receptor-specific antibodies and fluorochrome-conjugated reagents are diluted in PBS-gelatin or PBS-gelatin-Sap (for permeabilized cells) as required. Appropriate antibody dilutions should be determined empirically. **Note:** Cells should not be allowed to dry at any stage in the procedure.

3.4.1. Cells

1. Suspension cells: Approx. 3×10^6 cells for each experimental point.
2. Adherent cells: Cells are grown on sterile coverslips for 2 d. Cells should be about <50% confluent and well spread. If necessary, coverslips can be treated with adhesion molecules to help cells attach, or the cultures can be serum-starved for 12 h before the experiment to help cell spreading.

3.4.2. Chemokine Treatment

Adherent and suspension cells are treated with chemokines, as described in Method 1.

3.4.3. Fixation

Following treatment, cells are fixed in 1 mL of 3% PFA for 10 min, then washed in PBS and incubated in quench solution for at least 20 min.

Following fixation, all steps can be done at room temperature.

Attachment of suspension cells to coverslips facilitates labeling and washing. Fixed cells are placed on poly-D-lysine coated coverslips in 16-mm well plates for 30 min at room temperature. Unattached cells are removed by aspiration and the attached cells washed with PBS.

3.4.4. Labeling

For subsequent steps, adherent and suspension cells attached to coverslips are treated identically.

Cells are either stained intact, in which case only cell-surface receptors are stained, or after the plasma membrane is permeabilized, in which case both cell-surface and internal receptors will be stained (*see* **Note 6**). For intact cells, the cells should be washed and antibodies diluted in PBS-gelatin without Saponin or other detergents. For permeabilized cells, the cells should be washed and antibodies diluted in PBS-gelatin containing 0.05% Saponin (PBS-gel-Sap). Saponin should be present throughout the incubations and washes, except for the final wash before mounting. Detergent treatment may extract the receptors or destroy the antibody-binding sites for cell-surface chemokine receptors. We find Saponin is the most effective reagent for retaining cell-surface staining on permeabilized cells. However, for other antibodies different detergents may be effective.

1. Cells are incubated in 500 µL PBS-gelatin or PBS-gelatin-Sap (permeabilized cells) for 15 min to block nonspecific binding sites and, in the latter case, permeabilize the cells.
2. Cells are labeled in 250 µL of primary antibody diluted in PBS-gelatin or PBS-gelatin-Sap, for 30–60 min on a slow cycle reciprocal shaker. **Note:** If antibodies are in limited supply the coverslips can be inverted onto a 40–50 µL drop of diluted antibody on Parafilm. Care should be taken to prevent evaporation.
3. After staining the cells are washed 4 times (5 min for each wash) with PBS-gelatin or PBS-gelatin-Sap as appropriate.
4. Second layer: Repeat **steps 1** and **2** using appropriate second layer fluorescent antibody conjugates.
5. The coverslips are washed once in PBS and drained. The edges of the coverslips are blotted gently to remove surplus liquid, and the coverslips inverted on a drop of mountant on a clean glass slide. Coverslips should be stored in the dark while the mountant sets.

4. Notes

1. For suspension cells, the data can be normalized to cell number by counting an aliquot of the fixed cells after determining the ^{125}I activity. For adherent cells, the data can be normalized to cell protein by determining the protein content of each sample following γ counting. Using bicinchoninic acid (Pierce, Chester, UK), protein concentrations can be determined directly and are not affected by the NaOH content of the samples.
2. The media collected from cells after the 37°C incubation can be analyzed for the presence of TCA-soluble ^{125}I activity by precipitation with 10% TCA. The appearance of TCA-soluble activity can indicate delivery of the antibody to hydrolytic compartments of the endocytic pathway, such as lysosomes.
3. The protocol can be adapted to measure receptor recycling. If radiolabeled antibodies are internalized into cells by incubation at 37°C in the presence of chemokine or other agents, the acid-elution procedure can be used to remove radiolabeled antibodies remaining on the cell surface. If the acid-elution medium is not reduced below pH 3.0, and the washes kept brief and performed at 4°C, then the cells can be returned to 37°C culture (care should be taken to ensure that the endocytic-trafficking properties of cells are not perturbed by the low pH treatment). During a subsequent incubation at 37°C, receptors that recycle will return antibodies to the cell surface. These antibody molecules can be assessed by measuring the radioactivity that becomes accessible to a second round of acid elution.
4. Protocols similar to Method 2 are used: Antibodies are first bound to unfixed cells at 4°C, and the cells warmed to 37°C in the presence or absence of chemokine. At the required times, the cells are cooled to 4°C, fixed, and stained with specific fluorescent second reagents either intact or following permeabilization. Alternatively, the cell-surface antibodies can be removed by acid elution before fixation and staining.
5. Following internalization at 37°C in the presence of chemokine or other agents, the antibodies remaining on the cell surface are removed by acid elution. During a subsequent incubation at 37°C, recycling receptors return antibodies to the cell surface that become accessible to staining.
6. To investigate the subcellular distribution of internalized receptors, permeabilized cells can be co-stained with organelle-specific antibodies using essentially the same protocol. However, it is essential to ensure that the second reagents do not crossreact.

References

1. Ferguson, S. S., Barak, L. S., Zhang, J., and Caron, M. G. (1996) G-protein-coupled receptor regulation: role of G-protein-coupled receptor kinases and arrestins. *Can. J. Physiol. Pharmacol.* **74,** 1095–1110.
2. Ferguson, S. S., Downey III, W. E., Colapietro, A. M., Barak, L. S., Menard, L., and Caron, M. G. (1996) Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science* **271,** 363–366.

3. Goodman, O. B. J., Krupnick, J. G., Santinin, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) β-Arrestin acts as a clathrin adaptor in endocytosis of the β_2-adrenergic receptor. *Nature* **383,** 447–450.
4. Krueger, K. M., Daaka, Y., Pitcher, J. A., and Lefkowitz, R. J. (1997) The role of sequestration in G protein-coupled receptor resensitization. Regulation of beta2-adrenergic receptor dephosphorylation by vesicular acidification. *J. Biol. Chem.* **272,** 5–8.
5. Amara, A., Gall, S. L., Schwartz, O., Salamero, J., Montes, M., Loetscher, P., Baggiolini, M., Virelizier, J. L., and Arenzana-Seisdedos, F. (1997) HIV Coreceptor downregulation as antiviral principle: SDF-1alpha-dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. *J. Exp. Med.* **186,** 139–146.
6. Signoret, N., Oldridge, J., Pelchen-Matthews, A., Klasse, P. J., Tran, T., Brass, L. F., Rosenkilde, M. M., Schwartz, T. W., Holmes, W., Dallas, W., Luther, M. A., Wells, T. N. C., Hoxie, J. A., and Marsh, M. (1997) Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of the chemokine receptor CXCR4. *J. Cell Biol.* **139,** 651–664.
7. Solari, R., Offord, R. E., Remy, S., Aubry, J. P., Wells, T. N. C., Whitehorn, E., Oung, T., and Proudfoot, A. E. I. (1997) Receptor-mediated Endocytosis of CC-chemokines. *J. Biol. Chem.* **272,** 9617–9620.
8. Samanta, A. K., Oppenheim, J. J., and Matsushima, K. (1990) Interleukin 8 (monocyte-derived neutrophil chemotactic factor) dynamically regulates its own receptor expression on human neutrophils. *J. Biol. Chem.* **265,** 183–189.
9. Aramori, I., Zhang, J., Ferguson, S. S., Bieniasz, P. D., Cullen, B. R., and Caron, M. G. (1997) Molecular mechanism of desensitization of the chemokine receptor CCR-5: receptor signaling and internalization are dissociable from its role as an HIV-1 co-receptor. *EMBO J.* **16,** 4606–4616.
10. Mack, M., Luckow, B., Nelson, P. J., Cihak, J., Simmons, G., Clapham, P. R., Signoret, N., Marsh, M., Borlat, F., Stangassinger, F., Wells, T. N. C., Schlondorff, D., and Proudfoot, A. E. I. (1998) AOP-RANTES prevents CCR5 recycling: a novel inhibitory mechanism of HIV infectivity. *J. Exp. Med.* **187,** 1215–1224.
11. Barak, L. S., Ferguson, S. S., Zhang, J., Martenson, C., Meyer, T., and Caron, M. G. (1997) Internal trafficking and surface mobility of a functionally intact beta2-adrenergic receptor-green fluorescent protein conjugate. *Mol. Pharmacol.* **51,** 177–184.
12. Pelchen-Matthews, A., Armes, J. E., and Marsh, M. (1989) Internalization and recycling of CD4 transfected into HeLa and NIH-3T3 cells. *EMBO J.* **8,** 3641–3649.

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Chemokine Inhibition of HIV Infection

Jacqueline D. Reeves and Graham Simmons

1. Introduction

Human immunodeficiency virus (HIV) is a member of the retrovirus family, classified under the lentivirus genus. Retroviruses are enveloped RNA viruses, which contain a core of capsid proteins, viral RNA, and enzymes. All infectious retroviral virions contain an enzyme, reverse transcriptase, which catalyzes the formation of a complementary DNA strand from an RNA template. A double-stranded DNA copy of the viral RNA genome (proviral DNA) may then be integrated into and replicated with the host cell genome.

Viral and cellular lipid membranes must first fuse to allow entry of the viral core into a host cell. The primary receptor required for the entry of primate lentiviruses, HIV-1, HIV-2, and SIV, into cells is the CD4 molecule ***(1)***. The interaction of viral envelope protein with CD4 not only attaches virus particles to the cell surface but also induces conformational changes in the envelope protein. These structural alterations allow a secondary interaction with a coreceptor to occur which triggers the fusion process.

HIV isolates differ in their relative tropism for CD4-positive human cells, although all isolates infect activated primary CD4-positive T-lymphocytes. They can be broadly divided into two groups: (a) those that infect immortalized CD4-positive T-cell lines in vitro, induce syncytia (multinucleated giant-cells), and are termed syncytium inducing (SI), or T-cell tropic; and (b) those that infect macrophage cultures efficiently and do not induce syncytia in infected T-lymphocytes; these are called macrophage tropic or nonsyncytium-inducing (NSI) viruses. The majority of primary SI strains also infect macrophages and are described as dual-tropic viruses ***(2)***. Virus isolates passaged in peripheral blood mononuclear cells (PBMCs) are referred to as primary viruses, whereas those passaged in T-cell lines are designated T-cell line

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adapted (TCLA) viruses. NSI viruses are predominantly involved in transition and are usually present throughout disease. SI viruses emerge later on during disease progression, and are found in less than 50% of AIDS patients ***(3)***.

Levy and colleagues first showed in 1986 that soluble factors secreted by CD8+ T-lymphocytes were capable of inhibiting HIV infection of CD4+ T-cells ***(4)***. Nearly a decade later, Cocchi et al. ***(5)*** identified the β-chemokines RANTES, MIP-1α, and MIP-1β, as major components of CD8 cell secretions, which were able to inhibit infection by some HIV-1 strains. A few months later Feng et al. ***(6)*** discovered that a receptor with seven transmembrane domains (7tm), related to the chemokine receptor family was a coreceptor for SI HIV strains, but not for NSI strains. They termed this coreceptor "fusin," although following the discovery of its ligand, SDF-1, it is now termed CXCR4. It was then likely that a coreceptor for HIV strains blocked by RANTES, MIP-1α, and MIP-1β was a 7tm receptor for these β-chemokines and soon after several groups identified CCR5 as this coreceptor utilized by NSI HIV strains ***(7–9)***. At least ten other chemokine receptors and orphan 7tm receptor molecules have been shown to act as coreceptors for at least some HIV and SIV strains. These receptors include CCR1, CCR2b, CCR3, CCR4, CCR8, CX_3CR1, BOB (also known as gpr15), bonzo (also called STRL33 or TYMSTR), gpr1, and CCR8 ***(10–17)***. Ligands to coreceptors including chemokines, monoclonal antibodies (MAbs) and small organic molecules are able to block HIV entry and fusion, although inhibition is sometimes dependent on virus strain and cell type ***(18)***.

All primary NSI isolates use CCR5 as a coreceptor to CD4, although a minority can also utilize other coreceptors, for example CCR3. Primary SI and TCLA isolates, which are more fusogenic and replicate rapidly, use CXCR4 as a coreceptor. Many SI strains can also use a broad range of coreceptors in vitro including both CCR5 and CXCR4 ***(19,20)***. Recently a new nomenclature system has been proposed for viral phenotypes based on coreceptor usage ***(21)***. It is suggested that NSI strains using CCR5 are termed R5 viruses and SI viruses using CXCR4, but not CCR5, be termed X4 viruses. Dual-tropic SI strains using both CCR5 and CXCR4 will then be called R5X4 viruses.

In the past many groups exploited laboratory adapted strains of HIV, which have been extensively passaged through T-cell lines. The majority of such strains use CXCR4 exclusively and it is apparent that they have markedly distinct properties to primary HIV-1 isolates, including increased sensitivity to neutralization by soluble CD4, shedding of envelope proteins, and they often have defective accessory genes. Therefore, it is preferable to use primary viruses that have been isolated directly into PBMCs. Such isolates should be passaged as few times as possible, avoiding passage in cell lines.

A number of individuals frequently exposed to HIV, remain uninfected ***(22)***. Soon after the discovery of CCR5 as a coreceptor for NSI strains it was found that some of these exposed, uninfected individuals were homozygous for a mutation in their CCR5 genes ***(23–25)***. This mutation resulted in a 32 base-pair deletion in CCR5, that resulted in a premature stop codon and prevented cell surface expression. Individuals show no apparent adverse effects from being homozygous for this defective CCR5 gene. Also a mutation in the 3' non-coding region of SDF-1 has been reported to prolong the asymptomatic phase in HIV positive individuals as well as confer some protection against HIV infection ***(26)***. These findings have sparked a search for chemokines, chemokine antagonists, and small molecules able to specifically block HIV interaction, particularly via CCR5 as this seems to be the predominant coreceptor involved in transmission. Such reagents are candidates for use in contraceptives, as post exposure treatment, and as part of therapy for HIV infected individuals. A variant of RANTES, chemically modified at the N-terminus, termed AOP-RANTES, acts as an antagonist of RANTES-mediated chemotaxis ***(27)*** in primary monocytes. AOP-RANTES is a potent inhibitor of NSI HIV-1 infection not only in PBMCs, but also in primary macrophages and cell lines ***(27)***. AOP-RANTES has high affinity for CCR5 and induces internalization into endosomes more rapidly than RANTES. Moreover, unlike RANTES, AOP-RANTES prevents CCR5 from recycling from endosomes to the cell surface and thus acts to strip CCR5 irreversibly from the cell surface ***(28)***. Although the major coreceptor for macrophage infection is CCR5 ***(29)***, RANTES inhibits macrophage infection inefficiently ***(7)***. Differences in proteoglycan expression on macrophages compared to T-lymphocytes may partly explain this ***(30)***. Chemokines bind to proteoglycans present on the surface of T-cells and may thus be locally concentrated. AOP-RANTES, however, may have a sufficiently high affinity for CCR5 to overcome this phenomenon on macrophages, while RANTES does not. Several small molecules capable of blocking HIV interactions with CXCR4 have also been described ***(31–34)***.

Ligands specific for different chemokine receptors can also be used to assess which receptors are exploited by HIV-1 to infect primary cell types. Studying chemokine inhibition of primary cell types, such as PBMCs and macrophages, is important for determining which of the many chemokine receptors shown to be functional as HIV coreceptors are relevant for in vivo infection. For instance, PBMCs activated in vitro express a number of different 7tm coreceptors including CCR3, CCR5 and CXCR4 ***(35,36)***. Eotaxin, MIP-1β or SDF-1, ligands specific for CCR3, CCR5 and CXCR4 respectively, can thus be used in inhibition assays to assess if a particular HIV-1 strain uses any of these

coreceptors. It should be noted however that levels of inhibition do not necessarily equate to levels of coreceptor usage by a virus, for example He et al. ***(37)*** have shown that infection by R5R3 strains of microglia cells can be almost totally inhibited independently by either Eotaxin or MIP-1β. This may be due to aggregates of coreceptors at the cell surface.

Assaying inhibition of HIV infection can also be useful to determine the specificity of chemokines for particular receptors. For example vMIPII, a chemokine encoded by Human Herpes virus 8 (HHV8), was first shown to bind CCR3 by its ability to inhibit HIV infection of CCR3+ cells ***(38,39)***.

A number of approaches can be employed to study inhibition of HIV infection by chemokines. These are outlined in **Subheading 1.1.** to **1.5.**

1.1. Infection Using Cell-Free Virus

Both established cell lines and primary cells, such as PBMCs and cultured macrophages, can be used to study inhibition of HIV infection using cell-free virus. These assays first require the production of cell-free virus stocks (for methods *see* **ref. *40***). Cells can then be exposed to virus in the presence or absence of chemokines and assayed for virus replication 3–21 d post-infection. This can be done either by assaying release of new viral particles in the cell supernatant or by immunostaining cells for viral antigens. Thus when infectious viral particles are used, chemokine inhibition of both cell surface interactions and post-fusion events can be studied. For example the CCR4 ligand, MDC, has been reported to inhibit HIV-1 infection ***(41)***. As no HIV-1 strains have yet been shown to use CCR4 as a coreceptor it may be that its effect is downstream of viral entry. Disadvantages of studying chemokine inhibition using cell-free infection include the fact that cells have to be incubated for several days to allow viral replication to occur. Therefore, these assays are not as rapid as reporter gene and fusion assays. Further addition of ligands may be needed to prevent viral spread and amplification from cultures where infection is incompletely blocked by test reagents. Viral spread can also be reduced by adding AZT (Zidovudine) to infected cells (*see* **Note 5**).

Inhibition of cell-free virus infection can be carried out on a number of different cell types including primary cells, adherent cell lines, and T-cell lines. The majority of T-cell lines do not express CCR5 and are not infectable by R5 viruses. An exception is PM1 cells. Many of the initial chemokine inhibition assays were carried out on these cells, a T-cell line naturally expressing both CCR5 and CXCR4 ***(5)***. Both T-cell lines and primary cells may express a number of HIV coreceptors, and thus ligands to individual coreceptors may not block infection, if a virus can use an alternative coreceptor. It is therefore preferable to screen ligands initially on cell lines expressing CD4 and a single

defined coreceptor. Adherent cell lines expressing individual coreceptors can be obtained or constructed (*see* Chapter 10). However, while dual tropic R5X4 strains are efficiently inhibited by RANTES on CD4+ CCR5+ cell lines, it is more difficult to inhibit infection by CCR5 only using (R5) virus strains possibly due to high levels of CCR5 expression ***(38)***. Therefore, it is worthwhile screening compounds on primary PBMCs or PM1 cells where CCR5 is the predominant coreceptor used by these strains.

Methods for chemokine inhibition of cell-free virus infection on both primary and established cell lines are described in **Subheadings 2.1.** and **2.2.**

1.2. Infection of Cell Expressing Reporter Genes

As a quicker alternative to immunostaining for viral antigen following cell-free infection, cell lines expressing reporter genes can be used to give a result 1–2 d post-infection. For example, HeLa cells can be used stably expressing CD4, a coreceptor and containing a reporter construct with an HIV-1 LTR linked to the β-galactosidase gene (*LacZ*) ***(42)***. When infected by HIV, replication activates the LTR resulting in production of β-galactosidase. The speed of the result with this method also overcomes the problems of viral spread seen with cell-free virus infection. There are a limited number of suitable cell lines available however, and the assay does not function particularly efficiently with some primary HIV-1 strains. Methods for the inhibition of infection of *LacZ* reporter gene expressing cells are described in **Subheading 3.3.2.**

1.3. Virus Pseudotype Assays

The most common way to prevent viral spread from occurring is to ensure that only a single round of infection occurs by using pseudotypes. There are two forms of pseudotypes, both involving packaging of a reporter core within an HIV envelope. The first method uses a core from a highly cytopathic virus such as Vesicular Stomatitis Virus (VSV). Following HIV envelope-mediated entry, VSV replicates cytopathically and creates a plaque in the target cells. The second method involves packaging of a retrovirus gag/pol construct, bearing a reporter gene, within the HIV envelope. Following infection the reporter gene can then be used to assay infection. Either method gives a rapid result following infection. Inhibition assays with pseudotypes study only the effects on virus/cell membrane interactions, not post-entry stages of infection. However, this may be advantageous in dissecting the mechanisms of inhibition. One disadvantage of reporter gene pseudotypes is the possibility of relatively high background, particularly if used on primary cell types. Pseudotypes will not be further discussed here; for examples of reporter gene constructs *see* **refs.** ***42*** and ***47***.

1.4. Cell-to-Cell Fusion

T-cell lines chronically infected with virus will fuse and form syncytia when mixed with noninfected cells. Cell-to-cell fusion assays are quick to perform once chronically infected cell-lines are established, however are limited in that usually only TCLA viruses can be studied.

1.5. Envelope Fusion Assays

Another cell-to-cell fusion assay relies on the expression of cloned viral envelope proteins on the cell surface. Envelope expressing cells are mixed with target cells expressing relevant chemokine receptors. Fusion is then monitored either by syncytia formation or activation of a reporter gene within the target cells. These assays are usually completed overnight as compared to 2–21 d for cell-free infectivity assays. Once envelopes have been cloned, these assays can be performed in category 2 containment laboratories, while assays involving infectious virus require containment level 3 facilities. Unlike cell-to-cell fusion assays, primary virus envelopes can be studied, also envelopes can be directly cloned from patients thereby avoiding in vitro selection. However, as only a limited number of cloned envelopes are available, many more virus strains can be studied by cell-free infection assays. As with pseudotypes, fusion assays offer a useful method to study inhibition of cell surface interactions (or modulation of cell surface receptors), however they cannot be used to investigate inhibition of post entry stages of the viral life-cycle. Also, cell fusion does not necessarily equate to cell-free virus entry. For example, Bron et al. ***(43)***, have shown that the TCLA HIV-2 ROD envelope, when expressed on CHO cells, can utilize at least seven chemokine receptors to induce fusion. However, only some of these can act as coreceptors for cell-free infection by HIV-2 ROD ***(15,43)***. Many fusion assays result in expression of high concentrations of both HIV envelope and chemokine receptors and therefore may not be clinically relevant.

2. Materials

2.1. Chemokine Inhibition of HIV Infection of Primary PBMC and T-Cell Lines

1. Cells: PBMCs can be prepared from either fresh heparinized blood or leukocyte enriched buffy coats (*see* **Note 1**). A variety of CXCR4 expressing T-cell lines can be used for virus infectivity, for e.g., C8166. PM1 cells are a T-cell line that also naturally expresses CCR5 as well as CXCR4. Both cell lines are available from the NIH AIDS Research and Reference Reagent Program.
2. Cell culture medium: for PBMCs, RPMI 1640 (Gibco-BRL) containing 20% fetal calf serum (FCS), 60 μg/mL penicillin, and 100 μg/mL streptomycin. Supple-

ments required are phytohaemagglutinin (PHA) (Murex) and interleukin-2 (IL-2) (Boehringer Mannheim). Suspension cell lines are usually cultured in RPMI 1640 containing 10% FCS, 60 μg/mL penicillin and 100 μg/mL streptomycin.
3. Phosphate-buffered saline (PBS).
4. Lymphoprep (Nycomed) or Ficoll-isopaque (Pharmacia): for PBMC preparation.
5. Chemokines (commercially available from sources including PeproTech EC Ltd and R&D Systems).
6. Detection kit for HIV reverse transcriptase (RT) activity (e.g., Lenti-RT, Cavidi Tech., Upsala, Sweden) or p24 antigen (e.g., Coulter). Alternatively an "in house" P24 assay may be utilized ***(44)***.

2.2. Chemokine Inhibition of HIV Infection of Primary Macrophages and Adherent Cell Lines

1. Cells: macrophages can be prepared as described in ***(45)***. A variety of human and nonhuman cell lines stably expressing human CD4 and appropriate coreceptors can be used for chemokine inhibition assays. Some commonly used coreceptor expressing cell lines include the human glioma cell line, U87/CD4, the human osteosarcoma cell line, HOS/CD4, and the feline kidney cell line CCC/CD4 (available from the NIH AIDS Research and Reference Reagent Program). For *LacZ* reporter cell-free infection assays, HeLa-CD4-LTR-β-gal cells can be obtained from the NIH AIDS Research and Reference Reagent Program, or suitable cell lines can be generated.
2. Cell culture medium: macrophages are cultured in RPMI containing 10% human serum, 60 μg/mL penicillin, and 100 μg/mL streptomycin. Most adherent cells are cultured in Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL) containing 5–10% FCS, 60 μg/mL penicillin, and 100 μg/mL streptomycin.
3. Methanol/Acetone (1:1): stored at –20°C in a spark-proof freezer. Or 0.5% glutaraldehyde in PBS; for fixing *LacZ* reporter cells.
4. PBS containing 1% FCS (PBS/FCS).
5. Primary antibody: specific for a linear HIV epitope, for example we use a mixture of two murine anti-HIV-1 p24 antigen MAbs, 38:96K and EF7 ***(46)***, which recognize the majority of primary and T-cell line adapted HIV-1 strains. These MAbs are available from EU Programme EVA Centralised Facility and the Medical Research Council (MRC) AIDS Reagent Project (repository reference numbers EVA365 and EVA366).
6. Secondary antibody conjugated to β-galactosidase: for example, a β-galactosidase conjugated anti-mouse antibody (Southern Biotechnology; dilution 1:400).
7. β-galactosidase substrate: PBS containing 3 m*M* potassium ferricyanide, 3 m*M* potassium ferrocyanide, and 1 m*M* magnesium chloride, which can be stored at +4°C for approx 2 mo. 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal) should be added prior to use. X-gal is made up to 40 mg/mL in dimethyl formamide (DMF) and stored at –20°C in a polypropylene or glass container.

3. Methods

3.1. Chemokine Inhibition of HIV Infection of Primary PBMCs and T-Cell Lines

3.1.1. Preparation of PBMCs

1. Dilute blood or buffy coat 1 : 1 in RPMI (or PBS).
2. Carefully layer 2 vol of diluted blood onto 1 vol Lymphoprep and centrifuge at 700*g* for 30 min at room temperature.
3. Collect interphase and wash cells twice in RPMI (or PBS), centrifuging at 400*g* for 10 min.
4. Set cells up in a tissue culture flask at 3–5 × 10^6 cells/mL in growth medium containing 0.5 μg/mL PHA.
5. After 2–3 d, wash cells once in RPMI and resuspend at 1 × 10^6 cells/mL in culture medium containing 20 units/mL IL-2.
6. Incubate for a further 2–3 d before using in virus infectivity assays.

3.1.2. Cell-Free Infection Inhibition Assays

1. Add 1 × 10^5 PBMCs/well or 5 × 10^4 T-cell line cells/well (*see* **Note 2**) to a sterile V-bottomed 96-well microtiter plate and centrifuge at 400*g* for 5 min at room temperature.
2. Resuspend cells in 50 μL of culture medium containing relevant chemokine at 2X required final concentration and incubate for 30 min (or longer) at 37°C.
3. Add 50 μl of cell-free virus at 2X required final concentration (*see* **Note 3**) and incubate for 3 h (*see* **Note 4**) at 37°C.
4. Wash cells four times in culture medium, centrifuging at 400*g* for 5 minutes.
5. Resuspend in 200 μL culture medium (containing IL-2 for PBMCs) and relevant chemokine (*see* **Note 5**). Transfer to flat-bottomed 96-well tissue culture plate and incubate at 37°C.
6. Harvest 100 μL of supernatant every 2–3 d or at peak of infection and replenish with 100 μL fresh medium. Samples can either be assayed the same day or stored frozen (*see* **Note 6**).
7. Assay supernatants for p24 antigen or RT activity.

3.2. Chemokine Inhibition of HIV Infection of Primary Macrophages and Adherent Cell Lines

1. Adhere cells in 48-well trays (*see* **Note 7** for cell numbers per well) overnight.
2. Remove medium and add 75 μL of appropriate culture medium containing relevant chemokine at 2X required final concentration and incubate for 30 min or longer at 37°C.
3. Add 75 μL of cell-free virus at 2X required final concentration (*see* **Note 3**) and incubate for 3 h (*see* **Note 4**) at 37°C.
4. Remove chemokine/virus and wash cells three times in medium. Replace with 500 μL growth medium containing relevant chemokine (*see* **Note 5**).

5. Infection of adherent cell lines can be assayed by immunostaining (*see* **Subheading 3.3.**) and/or supernatants can be assayed for p24 antigen or RT activity. Macrophage culture medium should be changed every 4–5 d, up to day 30. Supernatants can be stored (*see* **Note 6**) for p24 or RT quantification to monitor a time course of infection. Alternatively, or in addition to p24 or RT analysis, macrophages can be immunostained for HIV antigens (*see* **Subheading 3.3.**).

3.3. Detection of Viral Replication in Adherent Cells

3.3.1. Immunostaining for Intracellular p24 Antigen

1. Most infected cell lines can be fixed and stained for p24 3–4 d after infection. Intracellular p24 accumulates more slowly after infection of macrophages and these cells are usually fixed 18–21 d post-infection. Cells are fixed for up to 10 min with 500 μL of cold methanol/acetone (1:1).
2. Remove methanol/acetone and wash cells once with 1 mL PBS/FCS.
3. Add 100 μL of primary antibody diluted in PBS/FCS. Incubate at room temperature for 1h.
4. Remove primary antibody and wash two times with 500 μL PBS/FCS.
5. Add 100 μL of β-galactosidase conjugated secondary antibody diluted in PBS/FCS. Incubate at room temperature for 1h.
6. Remove secondary antibody, wash once with 500 μL PBS/FCS and two times with 500 μL PBS alone.
7. Add 100 μL of β-galactosidase substrate and incubate at 37°C until color develops.
8. Determine number of blue-stained focus-forming units (ffu) of infection by light microscopy (*see* **Note 8**).

3.3.2. β-Galactosidase Detection in LacZ *Reporter Cells*

1. After 1–2 d incubation, wash cells in PBS.
2. Fix in 500 μL of 0.5% glutaraldehyde for 10 min.
3. Wash cells with 1 mL PBS.
4. Add 100 μL β-galactosidase substrate until blue color develops.
5. Determine number of blue-stained ffu of infection by light microscopy (*see* **Note 8**).

4. Notes

1. It is preferable but not essential to screen PBMCs from different donors to identify a donor whose cells replicate HIV efficiently and do not carry the Δ 32 CO25 mutation. Alternatively, leukocyte enriched buffy coats may be purchased from blood transfusion centers. Mixing PBMCs from two donors results in a mixed lymphocyte reaction which can increase cellular activation and hence HIV replication.
2. 5×10^4 suspension cells/well is based upon PM1 cells, a T-cell line that is sensitive to TCLA as well as non-TCLA isolates. Cell numbers should be adjusted according to the growth kinetics of the cell line chosen. For extended time course assays, cells may need to be divided, which is best performed at regular intervals.

3. Input virus should be standardized, ideally by calculating the tissue culture infectious dose (TCID) on the cell type to be assayed. Suitable virus doses to use are 100 or 1000 $TCID_{50}$s ($TCID_{50}$ is the dose required to give productive infection in 50% of test wells ***(47)***. Virus input can also be standardized to RT or p24 antigen levels; however, this may not directly correlate to infectious viral particles since any RT and p24 not incorporated into particles may be released by dying cells. Several dilution's of virus should also be tested as a high infectious dose may be less efficiently inhibited than a lower dose.
4. Virus can be left on cells for 2h to overnight, although levels of infection do not increase significantly after 3 h with most strains.
5. Although chemokines are thought to inhibit HIV infection at the point of cell entry, it is often not sufficient to have the chemokine present only at the time of the initial infection. Even after extensive washing, some virus which has been prevented from infecting cells will remain bound to the primary HIV receptor, CD4 (although bound virus can be reduced by treating the cells with trypsin before washing). Also, if only partial virus inhibition has occurred, any reduction of infection may be masked by viral spread during the assay. Therefore, it is advisable to include fresh chemokines following exposure to virus. For reasons of cost, the chemokine may be maintained only until the first time point (e.g. day 3) without adversely affecting the levels of inhibition for time courses of up to 12 d. Alternatively, virus spread can be reduced by adding AZT (5 μg/mL) to the cultures 24 h post infection, providing that AZT-resistant mutant viruses are not being evaluated.
6. Supernatants can be stored short term at –20°C. For long term storage, keep at –70°C.
7. The number of cells seeded the day before infection depends on cell type. Some examples of the numbers of cells to set up in a well of a 48-well tray (1-cm diam) are as follows: U87/CD4; 1×10^4, CCC/CD4; 5×10^4, RD/CD4; 2×10^4, HeLa/CD4; 2×10^4, HeLa-CD4-LTR-β-gal; $3–4 \times 10^4$. Five- to seven-d-old macrophages are set-up the day before infection at 1×10^5 cells/well of a 48-well tray.
8. Foci of infection resulting from cell-cell fusion, local viral spread, and division of infected cells can be estimated by carefully examining immunostained wells by light microscopy.

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References

1. Dalgleish, A. G., Beverley, P. C., Clapham, P. R., Crawford, D. H., Greaves, M. F., and Weiss, R. A. (1984) The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312,** 763–767.
2. Simmons, G., Wilkinson, D., Reeves, J. D., Dittmar, M. T., Beddows, S., Weber, J., et al. (1996) Primary, syncytium-inducing human immunodeficiency virus type

1 isolates are dual-tropic and most can use either Lestr or CCR5 as coreceptors for virus entry. *J. Virol.* **70,** 8355–8360.

3. Tersmette, M., de Goede, R. E., Al, B. J., Winkel, I. N., Gruters, R. A., Cuypers, H. T., et al. (1988) Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. *J. Virol.* **62,** 2026–2032.
4. Walker, C. M., Moody, D. J., Stites, D. P., and Levy, J. A. (1986) CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science* **234,** 1563–1566.
5. Cocchi, F., DeVico, A. L., Garzino Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P. (1995) Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* **270,** 1811–1815.
6. Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272,** 872–877.
7. Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., et al. (1996) HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381,** 667–673.
8. Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., et al. (1996) Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381,** 661–666.
9. Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M., and Berger, E. A. (1996) CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272,** 1955–1958.
10. Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., et al. (1996) The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85,** 1135–1148.
11. Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., et al. (1996) A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85,** 1149–1158.
12. Deng, H. K., Unutmaz, D., KewalRamani, V. N., and Littman, D. R. (1997) Expression cloning of new receptors used by simian and human immunodeficiency viruses. *Nature* **388,** 296–300.
13. Farzan, M., Choe, H., Martin, K., Marcon, L., Hofmann, W., Karlsson, G., et al. (1997) Two orphan seven-transmembrane segment receptors which are expressed in CD4-positive cells support simian immunodeficiency virus infection. *J. Exp. Med.* **186,** 405–411.
14. Liao, F., Alkhatib, G., Peden, K. W., Sharma, G., Berger, E. A., and Farber, J. M. (1997) STRL33, A novel chemokine receptor-like protein, functions as a fusion cofactor for both macrophage-tropic and T cell line-tropic HIV-1. *J. Exp. Med.* **185,** 2015–2023.

15. Reeves, J. D., McKnight, A., Potempa, S., Simmons, G., Gray, P. W., Power, C. A., et al. (1997) CD4-independent infection by HIV-2 (ROD/B): use of the 7-transmembrane receptors CXCR-4, CCR-3, and V28 for entry. *Virology* **231,** 130–134.
16. Loetscher, M., Amara, A., Oberlin, E., Brass, E., Legler, D. F., Loetscher, P., et al. (1997) TYMSTR, a putative chemokine receptor selectively expressed in activated T cells, exhibits HIV-1 coreceptor function. *Curr. Biol.* **7,** 652–660.
17. Horuk, R., Hesselgesser, R., Zhou, Y., Faulds, D., Halks-Miller, M., Harvey, S., et al. (1998) The CC chemokine I-309 inhibits CCR8-dependent infection by diverse HIV-1 strains. *J. Biol. Chem.* **273,** 386–391.
18. McKnight, A., Wilkinson, D., Simmons, G., Talbot, S., Picard, L., Ahuja, M., et al. (1997) Inhibition of human immunodeficiency virus fusion by a monoclonal antibody to a coreceptor (CXCR4) is both cell type and virus strain dependent. *J. Virol.* **71,** 1692–1696.
19. Connor, R. I., Sheridan, K. E., Ceradini, D., Choe, S., and Landau, N. R. (1997) Change in coreceptor use correlates with disease progression in HIV-1-infected individuals. *J. Exp. Med.* **185,** 621–628.
20. Rucker, J., Edinger, A. L., Sharron, M., Lee, B., Berson, J. F., Yi, Y., et al. (1997) Utilization of chemokine receptors, orphan receptors, and herpes virus-encoded receptors by diverse human and simian immunodeficiency viruses. *J. Virol.* **71,** 8999–9007.
21. Berger, E. A., Doms, R. W., Fenyo, E.-M., Korber, B. T. M., Littman, D. R., Moore, J. P., et al. (1998) A new classification for HIV-1. *Nature* **391,** 240.
22. Detels, R., Liu, Z., Hennessey, K., Kan, J., Visscher, B. R., Taylor, J. M., et al. (1994) Resistance to HIV-1 infection. Multicenter AIDS Cohort Study. *J. Acquir. Immune Defic. Syndr.* **7,** 1263–1269.
23. Liu, R., Paxton, W. A., Choe, S., Ceradini, D., Martin, S. R., Horuk, R., et al. (1996) Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86,** 367–377.
24. Dean, M., Carrington, M., Winkler, C., Huttley, G. A., Smith, M. W., Allikmets, R., et al. (1996) Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. *Science* **273,** 1856–1862.
25. Samson, M., Libert, F., Doranz, B. J., Rucker, J., Liesnard, C., Farber, C. M., et al. (1996) Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* **382,** 722–725.
26. Winkler, C., Modi, W., Smith, M. W., Nelson, G. W., Wu, X., Carrington, M., et al. (1998) Genetic Restriction of AIDS Pathogenesis by an SDF-1 Chemokine Gene Variant. *Science* **279,** 389–393.
27. Simmons, G., Clapham, P. R., Picard, L., Offord, R. E., Rosenkilde, M. M., Schwartz, T. W., et al. (1997) Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science* **276,** 276–279.
28. Mack, M., Luckow, B., Nelson, P. J., Cihak, J., Simmons, G., Clapham, P. R., et al. (1998) Aminooxypentane-RANTES induces CCR5 internalization but inhibts recycling: A novel inhibitory mechanism of HIV infectivity. *J. Exp. Med.* **187,** 1215–1224.

29. Connor, R. I., Paxton, W. A., Sheridan, K. E., and Koup, R. A. (1996) Macrophages and CD4+ T lymphocytes from two multiply exposed, uninfected individuals resist infection with primary non-syncytium-inducing isolates of human immunodeficiency virus type 1. *J. Virol.* **70,** 8758–8764.
30. Oravecz, T., Pall, M., Wang, J., Roderiquez, G., Ditto, M., and Norcross, M. A. (1997) Regulation of anti-HIV-1 activity of RANTES by heparan sulfate proteoglycans. *J. Immunol.* **159,** 4587–4592.
31. Doranz, B. J., Grovit Ferbas, K., Sharron, M. P., Mao, S. H., Goetz, M. B., Daar, E. S., et al. (1997) A small-molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 coreceptor. *J. Exp. Med.* **186,** 1395–1400.
32. Donzella, G. A., Schols, D., Lin, S. W., Este, J. A., Nagashima, K. A., Maddon, P. J., et al. (1998) AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. *Nat. Med.* **4,** 72–77.
33. Schols, D., Este, J. A., Henson, G., and De Clercq, E. (1997) Bicyclams, a class of potent anti-HIV agents, are targeted at the HIV coreceptor fusin/CXCR-4. *Antiviral Res.* **35,** 147–156.
34. Murakami, T., Nakajima, T., Koyanagi, Y., Tachibana, K., Fujii, N., Tamamura, H., et al. (1997) A small molecule CXCR4 inhibitor that blocks T cell line-tropic HIV-1 infection. *J. Exp. Med.* **186,** 1389–1393.
35. Bleul, C. C., Wu, L., Hoxie, J. A., Springer, T. A., and Mackay, C. R. (1997) The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proc. Natl. Acad. Sci. USA* **94,** 1925–1930.
36. Sallusto, F., Mackay, C. R., and Lanzavecchia, A. (1997) Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science* **277,** 2005–2007.
37. He, J., Chen, Y., Farzan, M., Choe, H., Ohagen, A., Gartner, S., et al. (1997) CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. *Nature* **385,** 645–649.
38. Kledal, T. N., Rosenkilde, M. M., Coulin, F., Simmons, G., Johnsen, A. H., Alouani, S., et al. (1997) A broad-spectrum chemokine antagonist encoded by Kaposi's sarcoma-associated herpesvirus. *Science* **277,** 1656–1659.
39. Boshoff, C., Endo, Y., Collins, P. D., Takeuchi, Y., Reeves, J. D., Schweickart, V. L., et al. (1997) Angiogenic and HIV-inhibitory functions of KSHV-encoded chemokines. *Science* **278,** 290–294.
40. Tersmette, M., Koot, M., De Goede, R. E. Y., Kootstra, N., and Schuitemaker, H. (1995) Isolation and biological characterization of primary HIV-1 isolates, in *HIV: A Practical Approach* (Karn, J., ed.), vol. 1, Oxford University Press, Oxford, UK, pp. 47–61.
41. Pal, R., Garzino Demo, A., Markham, P. D., Burns, J., Brown, M., Gallo, R. C., and DeVico A. L. (1997) Inhibition of HIV-1 infection by the beta-chemokine MDC. *Science* **278,** 695–698.
42. Kimpton, J. and Emerman, M. (1992) Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. *J. Virol.* **66,** 2232–2239.

43. Bron, R., Klasse, P. J., Wilkinson, D., Clapham, P. R., Pelchen Matthews, A., Power, C., et al. (1997) Promiscuous use of CC and CXC chemokine receptors in cell-to-cell fusion mediated by a human immunodeficiency virus type 2 envelope protein. *J. Virol.* **71,** 8405–8415.
44. Moore, J. P., McKeating, J. A., Weiss, R. A., and Sattentau, Q. J. (1990) Dissociation of gp120 from HIV-1 virions induced by soluble CD4. *Science* **250,** 1139–1142.
45. Collins, M., Montaner, L. J., Herbein, G., and Gordon, S. (1995) HIV infection of macrophages, in: *HIV: A Practical Approach* (Karn, J., ed.), vol. 1, Oxford University Press, Oxford, UK, pp. 63–76.
46. Hinkula, J., Rosen, J., Sundqvist, V. A., Stigbrand, T., and Wahren, B. (1990) Epitope mapping of the HIV-1 gag region with monoclonal antibodies. *Mol. Immunol.* **27,** 395–403.
47. McKnight, A., Clapham, P. R., and Schulz, T. F. (1995) Detection of HIV entry into cells, in: *HIV: A Practical Approach* (Karn, J., ed.), vol. 1, Oxford University Press, Oxford, UK, pp. 129–141.

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The Production of Chemokine Specific Monoclonal Antibodies

Chemokine RANTES

Peter J. Nelson

1. Introduction

Monoclonal antibodies (MAbs) produced through the technique of somatic cell fusion are fundamental tools in the exploration of chemokine biology. This chapter details the procedures that were used to generate a panel of MAbs directed against the human chemokine RANTES ***(1,2)***. The general approach described here should be broadly applicable to the generation of MAbs directed against other members of this cytokine family.

The methods leading to the production of hybridomas and their monoclonal antibody (MAb) products are well established. An excellent detailed review of the procedure by Yokoyama is found in Current Protocols in Immunology ***(3)***. The overall process involves the immunization of mice, B-cell fusion with a hybridoma partner, the growth of the hybridoma with cloning through limiting dilution, the propagation of stable hybridomas, screening for antibody specificity, and finally, a study of the application and function of antibody binding activity. This process is labor intensive and several months are needed before appropriate hybridoma cell lines are obtained and the resultant monoclonal antibodies fully characterized. However, the results frequently yield a series of highly specific MAbs, available in unlimited quantities, that are often directed against different epitopes of chemokine protein ***(1,2,3)***.

1.1. RANTES Protein

RANTES (Regulated upon Activation Normal T cell Expressed and Secreted) is a member of the C-C chemokine subfamily. The RANTES protein is composed of 91 amino acids, including a 23 amino acid leader sequence

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cleaved in the endoplasmic reticulum. The "mature" protein is a highly basic polypeptide (PI~9.5) with a molecular mass of approx 8 kilodaltons that is probably a dimer at physiological pH and protein concentrations ***(4)***. This protein acts as a chemotactic agent, but it can also activate leukocyte subpopulations ***(4)***. RANTES can also inhibit the replication of some strains of HIV-1 through interaction with CCR5, an important coreceptor for viral entry into cells, and one of several chemokine receptors that binds RANTES ***(5)***.

The generation of human RANTES specific MAbs has allowed the investigation of RANTES protein expression in histological and cytological preparations ***(1,4,6)***. RANTES protein expression is seen in vivo in diseases characterized by a mononuclear cell infiltration such as renal allograft rejection, delayed-type hypersensitivity, kidney glomerular disease, and inflammatory lung disease ***(4)***. These specific antibody reagents have also been applied to the detection and quantitation of RANTES antigen using techniques that include: quantitative RANTES ELISA, immunoprecipitation, Western blot, and the study of internal protein stores by fluorescence activated cell sorting (FACS) analysis ***(2)***. In addition, several of the MAbs were found to block RANTES induced chemotactic activity and thus can serve as important controls in assessing RANTES induced bioactivity.

2. Materials

2.1. Recombinant RANTES Protein

In general, the production of good MAbs depends to a large part upon the quality, purity and availability of antigen. In the production of the anti-RANTES chemokine monoclonal antibodies described here, a pET-3 *E. coli* expression system was used that allowed the manufacture of large quantities of recombinant human RANTES protein (*see* **Notes 1**, **2**, and **3**). The recombinant protein was also used as a positive control in Western and immunoprecipitation experiments performed at later stages of the characterization of the MAbs ***(1)***.

1. Standard pathogen free animal facilities for housing and handling of animals ***(3)***.
2. Preimmune bleeds of the mice should be taken before the procedure is started to establish the background binding of serum to specific antigen.
3. Denatured recombinant protein was used as immunogen. It was injected both with, and without adjuvant ***(3)***. The denatured protein ensured that multiple epitopes of the protein were available for presentation. However, the renatured protein was eventually used to screen the hybridomas.
   a. The mice should be kept in a pathogen free environment. (Retired breeder mice (C57/blk6) were used.)
   b. Complete Freunds adjuvant (Sigma) (*see* **Note 5**).
   c. Incomplete Freunds adjuvant (Sigma) (*see* **Note 5**).

   d. Three-mL glass syringes with 19- and 21-gauge needles, double-ended locking hub connector (Luer-Lok, Becton Dickinson) used to generate the adjuvant-antigen emulsion ***(3)***.
4. Standard tissue culture facilities, including sterile hood.
5. 50-mL disposable polypropylene centrifuge tubes.
6. Beckman (Fullerton, CA) TH4 rotor or equivalent.
7. 175-cm^2 flasks.
8. Fine mesh screen.
9. 50% polyethyene glycol (sterile).
10. Dimethyl sulfoxide (DMSO).
11. Nunc Immuno Plates (MaxiSorp F96).
12. Sp2 fusion hybridoma partner cells, obtained from the ATCC, and cultured as per ATCC directions.
13. Hybridoma growth media (HAT) (Gibco-BRL).
14. RPMI media and fetal calf serum (Gibco-BRL).
15. Phosphate-buffered saline (PBS).
16. ATBS reagents for the detection of horseradish peroxidase (HRP) activity were obtained from Boehringer Mannheim (Cat. No. 1 112 422 and 1 112 597) and were used according to the manufacturer's directions.
17. Refolded protein was used as the capture antigen during hybridoma screening (*see* **Notes 2** and **3**). It is also possible to perform a second screen using the denatured protein antigen in order to screen for antibodies that bind masked epitopes within the protein. The resultant MAbs should work for Western blot analysis.

3. Methods

3.1. Immunization

Mice were immunized twice a week for 3 wk with 10 μg of RANTES protein for each injection.

1. Purified, denatured recombinant human RANTES was used as an immunogen to inject 5 mice intramuscularly (*see* **Note 6**).
2. Week 1: the mice were injected with RANTES protein in Complete Freunds Adjutant ***(3)***. Three days later the animals were reinjected with 10 μg of denatured RANTES protein (no adjuvant).
3. Week 2: Incomplete Freunds Adjuvant with RANTES protein was used as the immunogen ***(3)***, followed three days later by a boost of 10 μg of denatured RANTES protein (no adjuvant).
4. Week 3: protocol was a repeat of the injection procedure used in week 2.
5. One week later the animals were bled, and serum was prepared from whole blood and tested in ELISA. (If the sera was negative in the direct ELISA, then the animals would have been reinjected for two additional weeks and then retested with ELISA.)
6. "Positive titer" animals (all five) were then used for hybridoma fusion.

3.2. Hybridoma Fusion

1. After 4 wk, the popliteal, inguinal, and axilliary lymph nodes were removed from the mice. The thymus was also removed for use as feeder cells for the hybridomas.
2. The nodes and thymus were then placed in 1X PBS (or RPMI), teased apart on glass slides (or dishes), and filtered through a fine mesh screen. Clumps of tissue were allowed to settle after filtration and were not used for the subsequent fusion.
3. The resultant cell suspension was then transferred into 15-mL Falcon tubes, washed with 1X PBS or RPMI, and the cells were counted.
4. The Sp2 fusion partner cells were washed in a 50-mL tube and counted.
5. The Sp2 cells and lymph node cells were then combined at a ratio of 1:1 and spun together in a 50-mL tube at 500*g* for 5 min.
6. The supernatant was decanted, the pellet resuspended, and 1 mL of fusion media containing: 40% polyethylene glycol (PEG), 10% DMSO, in RPMI medium (no FCS) was added slowly over 30 s with continuous gentle tapping to suspend the cells.
7. The mixture was allowed to stand for 2 min, then 0.25 mL of 5% DMSO, in RPMI medium (no FCS) was then added every 15 s for a total of 10 min. Then PBS was added to a total of 50 mL, and the tubes were spun at room temperature.
8. The supernatant was decanted and excess medium removed with a sterile cotton tip applicator. Ten milliliters of RPMI followed by 30 mL of fusion medium were combined and used to fill two 96-well Limbro flat-bottom plates. Cells isolated from approximately one thymus were added to each 96-cell plate as feeders.
9. 200 μλ/well of the fusion suspension was added to each well of the 96-cell plate. Each well contained approx 2×10^5 fusion cells.
10. The cells were fed every 7 d with hypoxanthine-containing medium. Positive fusions were generally testable within 2 wk.

3.3. Screening of Hybridomas

1. Fusion hybrids were screened by ELISA on Nunc Immuno Plates (MaxiSorp F96) using refolded recombinant RANTES as the capture antigen (*see* **Notes 2** and **3**).
2. 20 μg per mL of RANTES protein in PBS pH 7.4, was incubated (100 μL per well) over night on the 96-well plates (Nunc Immuno Plates, MaxiSorb F96). The plates were washed twice with 1X PBS and blocked for 2 h at room temperature using 1% BSA in 1X PBS (200μL per well). The plates were washed 4 times using 1X PBS and then used to test for positive hybridomas.
3. 50 to 100 μυλ of hybridoma supernatant were added to each well and incubated at room temperature for 1 h (appropriate antibody controls were bound directly to the plate). The plates were washed 4 times with 1X PBS.
4. The ELISAs were developed using HRP conjugated goat-antimouse immunoglobulin antibody as the secondary antibody (the appropriate dilution of the second-

ary antibody should be determined empirically). The secondary antibody was added and allowed to bind for 1 h at room temperature.
5. The plates were washed 4 times and the HRP activity detected using ATBS reagents.
6. Positive hybridomas were transferred to larger wells, additional feeder cells were provided, the samples were retested, and then subcloned by limiting dilution (*see* **Note 7**).
7. The isotypes of selected MAbs were determined using a commercial mouse MAb typing kit (Amersham, Amersham, UK).
8. The characterization of four antihuman MAbs produced in this manner are detailed by von Luettichau et al. ***(1)*** and Krensky and Nelson ***(2)***.

4. Notes

1. To express recombinant RANTES protein using the pET expression approach, the coding sequence of human RANTES (with the signal sequence deleted) was cloned into the *E. coli* expression vector pET-3 ***(7)***. The RANTES/pET-3 construct was then transfected into the *E. coli* strain BL21(De)3, grown, and then induced with IPTG. It should be determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) what distribution of the resultant recombinant protein is sequestrated in bacterial inclusion bodies ***(7,8)***. (While inclusion bodies can facilitate protein purification, it will require subsequent denaturation and protein refolding steps ***[8,9]***.)
2. It was determined that the recombinant RANTES protein produced in the pET-3 expression system was localized almost exclusively to bacterial inclusion bodies. This allowed easy purification essentially as described in **refs. *1,2***.
3. To refold inclusion body/RANTES protein, the protein was first denatured in 6 *M* guanidine and then slowly injected into refolding buffer (50 m*M* Tris-HCl, pH 7.0, 50 m*M* KCl, 0.1 m*M* EDTA, 0.75 *M* arginine, 12 m*M* glutathione [reduced form], and 1.2 m*M* glutathione [oxidized form]) to reach a final concentration of guanidine of 1 *M*. The mixture was gently stirred at 4°C overnight. The resultant protein solution was then subjected to dialysis against 20 m*M* acidic acid, pH 3.0, followed by dialysis against 10 m*M* MOPS, pH 7.0, containing 100 m*M* NaCl. Precipitated, misfolded, aggregated protein was removed by centrifugation for 30 min at 100,000*g*. The protein sample was then passed over a BioRex ion exchange column (Bio-Rad Laboratories, Richmond, CA) equilibrated in 10 m*M* MOPS, pH 7.0, 100 m*M* NaCl, washed with 10 m*M* MOPS, pH 7.0, 500 m*M* NaCl, and eluted with a 10 m*M* MOPS pH 7.0, 0.5–2 *M* NaCl linear gradient. The RANTES protein eluted at approx 1.5 *M* NaCl. The $O.D._{280}$was determined, peak fractions pooled and concentrated using PEG (Aquacide II, Calbiochem, La Jolla, CA) Centraprep, and Centracon concentrators (Amicon, Beverly, MA) and further purified using fast performance liquid chromatography (FPLC) and a Superdex 75 2560 column developed with 1.5 *M* NaCl, 10 m*M* MOPS, pH 7.0, (Pharmacia, Uppsala, Sweden). The resultant recombinant

protein was tested for endotoxin contamination by Limulus assay and if required passed through a Detoxi gel column (Sigma, St. Louis, MO). Yields were typically between 25 and 35 mg of refolded protein/1 L starting material.

4. Due to aggregation at high protein concentrations (over one mg/mL) the RANTES protein was stored in high salt (1.5 *M* NaCl) concentrations or at low pH (pH 2.5).
5. Complete and Incomplete Freunds adjuvant were used in this procedure. These agents are extremely inflammatory. Always use protective eyewear and gloves when handling these agents. Several excellent alternative adjuvants that are far less stressful to the animals have been recently developed ***(3)***. The use of these new agents should be strongly considered.
6. Mice were injected with purified recombinant protein that was first denatured in 6 *M* guanidine, followed by dialysis in 1X PBS.
7. In production of the anti-RANTES monoclonal antibodies, after generation of the hybridoma fusions, plate ELISA was performed on 480 hybridomas. After repeated subcloning using limiting dilution analysis, four reproducibly positive hybridomas were characterized.
8. Cross-reactivity of the antichemokine monoclonal antibodies with other chemokine family members was tested using plate ELISA and Western-blot analysis. In the Western-blot analysis, a blotting apparatus (Bio-Rad) was used to bind a series of control chemokines and cytokines to Immobilon-P (Millipore, Bedford, MA) transfer membranes. The MAbs, used at a 1:100 dilution of spent hybridoma supernatant, were tested for cross-reactivity. The results should demonstrate strong binding to the recombinant protein and show no cross-reactivity with the panel of control chemokines. In the control ELISA experiments, titrations representing one microgram to one nanogram of different chemokine proteins were bound directly to Nunc-Immuno Plates (MaxiSorb) and developed using horseradish peroxidase conjugated goat-antimouse immunoglobulin antibody as the secondary antibody ***(1,2)***.

References

1. von Luettichau, I., Nelson, P. J., Vandereijin, M., Huie, P., Pattison, J. M., Stahl, R. A. K., et al. (1996) RANTES chemokine expression in normal and diseased tissue. *Cytokine* **8,** 89–98.
2. Krensky, A. M. and Nelson, P. J. (1997) Expression of the chemokine RANTES and production of monoclonal antibodies. *Methods Enzymol.* **287,** 162–174.
3. Yokoyama, W. M. (1991) Production of monoclonal antibodies, in *Current Protocols in Immunol.* (Coligan, J. E., Kruisbeeck, A. M., Marguilies, D. H., Shevach, E. M., and Storober, W., eds.), John Wiley and Sons, pp. 2.5.1–2.5.17.
4. Nelson, P. J., Pattison, J. M., and Krensky, A. M. (1998) The chemokine RANTES, in *Cytokines* (Handbook of Immunopharmacology Series) (Thorpe, R. and Mire-Sluis, A., eds.), Academic, pp. 433–448.
5. Nelson, P. J. and Krensky, A. M. (1998) Chemokines, lymphocyte biology and viruses: what goes around, comes around. *Curr. Opin. Immunol.* **10(3),** (in press).

6. Pattison, J. M., Nelson, P. J., Huie, P., von Luettichau, I., Farshid, G., Sibley, R. K., and Krensky, A. M. (1994) RANTES chemokine is highly expressed in cell mediated transplant rejection of the kidney. *Lancet* **343,** 209–211.
7. Studier, F. W. and Moffatt, B. A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189,** 113–130.
8. Kelly, R. F. and Winkler, M. E. (1990) Folding of eukaryotic proteins produced in *Escherichia coli*. *Genetic Engineering* **12(1),** pp. 1–9.
9. Furuta, R., Yamagishi, J., Kotani, H., Sakamoto, F., Fukui, T., Matsui, Y., et al. (1989) Production and characterization of recombinant human neutrophil chemotactic factor. *J. Biochem.* **106,** 436–441.

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Monoclonal Antibodies to Chemokine Receptors

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1. Introduction

Monoclonal antibodies (MAbs) to chemokine receptors are providing remarkable insight into the roles these receptors play in basal leukocyte trafficking for immune response and surveillance as well as cell specific recruitment to sites of inflammation. This latter role in particular has established chemokine receptors as attractive targets for novel antiinflammatory drug discovery. The large number of chemokines and receptors, the complex nature of their distinct and overlapping binding specificities, and the differential expression of receptors on leukocytes may provide the opportunity to develop therapeutics that selectively target subsets of leukocytes and thus avoid the broad immunosuppressive actions of most currently available antiinflammatory drugs ***(1)***. The major obstacle in the path to such therapeutics is unlikely to be the discovery and development of potent and selective chemokine receptor antagonists, inasmuch as seven transmembrane spanning G-protein couple receptors have proven to be a most successful class of drug targets ***(2)***. However, sorting through the various and complex functions mediated by a multitude chemokine receptor-ligand interactions in order to ascertain which receptor(s) is responsible for site specific recruitment of cells during initiation, progression, and maintenance of a particular disease will be a formidable task.

MAbs make excellent tools with which to begin elucidating the relationship between chemokine, receptor, leukocyte recruitment, and disease. Flow cytometric analysis of chemokine receptor expression on peripheral blood leukocytes together with immunohistochemical examination of chemokine receptor expression on leukocytes recruited to inflammatory lesions may be used to correlate specific receptors with disease associated leukocyte infiltrates.

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Immunoassay or immunohistochemical assessment of ligands associated with these sites may confirm or further define an association. mAbs to CCR5 and CXCR3, for example, were used to show that these receptors mark a subset of peripheral blood T cells recruited to the synovium of rheumatoid arthritis patients as well as to chronic vaginitis and ulcerative colitis tissues ***(3)***. CCR3 specific MAbs demonstrated that eosinophils predominately use this single receptor to mediate chemokine actions ***(4,5)***, which, together with the enhanced expression of CCR3 ligands in asthmatics, has marked this receptor as a prime therapeutic target ***(6)*** for blockade of eosinophil migration into the lung.

Definitive linkage of a specific receptor to the initiation or maintenance of a disease, however, is difficult to obtain. Most leukocytes, particularly lymphocytes, coexpress several chemokine receptors, the levels of which may be modulated by various cytokines and stimuli ***(7–9)***. Recent experiments demonstrating that leukocytes can sequentially migrate through at least two (and most likely more) spatially distinct chemoattractant gradients suggests that several chemokine receptors may be used, if not required, for targeting cells to a specific site ***(10)***. In fact, different receptors may be utilized to mediate distinct functions such as integrin activation for firm adhesion, extravasation through blood vessel walls, tissue navigation, site-specific arrest, and initiation of effector functions. This hypothesis is supported by the fact that most inflammatory lesions generate numerous chemokines, many of which bind and signal through more than one receptor, as well as data demonstrating that different chemokine receptors on the same cell or cell type may perform discrete functions in response to the same or overlapping set of chemokines ***(11–13)***.

Modified chemokine-derived antagonists ***(14,15)*** and neutralizing antisera and MAbs against chemokines ***(16–20)*** have provided compelling evidence in several animal models that disruption of chemokine-receptor interactions can prevent disease or delay disease onset. It was further demonstrated in some models that this inhibition can alleviate symptoms and prevent further tissue destruction even after disease is established. With these reagents, however, it is difficult to elucidate which receptor(s) is responsible for disease and at what point(s) during disease progression blockade of a specific chemokine-receptor interaction is efficacious. This is due primarily to the promiscuous nature of receptor binding by many chemokines ***(21)*** and the fact that some chemokine modifications have been shown to alter receptor selectivity ***(22,23)***. In addition, the high degree of amino acid sequence similarity between some chemokines makes it difficult to generate neutralizing antisera which does not cross-react to some extent with other ligands. Alternatively, ligand blocking MAbs to chemokine receptors may prove to be the ideal proof of concept tools for defining the role of a specific receptor in a particular disease and validating it as therapeutic target.

As the number of chemokines and receptors continue to expand so too does the list of ascribed biological functions. Beyond their characterized roles in leukocyte trafficking and inflammation, chemokines have been shown to effect angiogenesis ***(24–26)***, hematopoiesis ***(27–29)***, T-cell differentiation ***(30)***, apoptosis ***(31)***, and viral infection ***(32,33)***, although the biological significance of these effects as well as the mechanism of action remain to be determined in many cases. Furthermore, chemokine receptor expression has been reported on nonlymphoid cell types in brain and vasculature ***(34,35)***. Antibodies will no doubt prove to be useful in determining the function of these receptors in other systems.

Generation of monoclonal antibodies to chemokine receptors, particularly blocking antibodies, has generally been considered a difficult endeavor. This is due in part, perhaps, to the small portion of total receptor actually exposed on the cell surface. With an average length of about 360 amino acids and ranging in size from 350–379 amino acids, chemokine receptors span the cell membrane seven times leaving only three relatively short extracellular loops ranging in size from about 15–30 amino acids and an amino terminal extracellular domain varying in length from about 30–50 amino acids to serve as immunogen. The amount of exposed receptor to which antibodies may bind is further reduced by two disulfide bonds linking together extracellular loops 1 and 2 and the amino terminal domain with extracellular loop 3, thus making some amino acids inaccessible to antibody. Low expression levels and the glycosylation of extracellular domains most certainly compound the problem for some receptors. Nevertheless, specific MAbs, many of them blocking, have been generated to most chemokine receptors including CXCR1 and CXCR2 ***(11,36,37)***, CXCR3 ***(3)***, CXCR4 ***(38)***, CXCR5 ***(39)***, CCR2 ***(40)***, CCR3 ***(4,5)***, CCR5 ***(41,42)***, among several others.

Two general methods are described below which have been used successfully by several laboratories to generate MAbs to chemokine receptors. The first method describes the use of synthetic peptides as immunogen with the sequence derived from the amino terminal domain of the receptor. The second method describes the use of chemokine receptor transfectants as immunogen. High levels of surface receptor expression are essential to the success of this latter method. Protocols for making receptor transfectants may be found in **Chapter 10**.

2. Materials

1. Dulbecco's Modified Eagle's Medium (DMEM, Gibco-BRL).
2. Phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Gibco-BRL).
3. 1 *M* HEPES, pH 7.4 (Gibco-BRL).
4. Fetal calf serum (FCS; Gibco-BRL).

5. Hypoxanthine, Aminopterin, and Thymidine supplement (100X HAT, Sigma).
6. Hypoxanthine and Thymidine supplement (100X HT, Sigma).
7. Sodium pyruvate, Pen-Strep, and L-glutamine (Gibco-BRL).
8. Polyethylene glycol (PEG 4000; sterile 50% solution, Merck).
9. SP2/0-Ag14 or P3X63Ag8.653 myeloma fusion partner (ATCC).
10. Ammonium chloride solution: 0.02 *M* Tris-HCl, pH 7.2, 0.14 *M* NH_4Cl.
11. Pathogen free mice, 6–8 wk-old (Charles River).
12. 175-cm^2 tissue-culture flasks.
13. 15- and 50-mL conical centrifuge tubes.
14. 96-well flat-bottom tissue culture plates.
15. 1- and 3-mL syringes.
16. 22- and 25-gauge needles.
17. 110-mm^2 tissue-culture Petri dishes.
18. FITC-conjugated goat antimouse IgG (Jackson ImmunoResearch).
19. FACS buffer: PBS plus 2% FCS, 0.01% sodium azide.

2.1. Peptides as Immunogens

1. Synthetic peptide (commercial sources include American Peptide Company, Sunnyvale, CA and Severn Biotech, Kidderminster, UK).
2. Synthetic peptide coupled to carrier such as KLH (Keyhole Limpet Hemocyanin) or PPD (Purified Protein Derivative of tuberculin).
3. Complete Freund's Adjuvant (CFA; Sigma).
4. Incomplete Freund's Adjuvant (IFA; Sigma).
5. 3-way stopcock.
6. 96-well, NUNC-ImmunoTM plates (Flow Labs).
7. Carbonate buffer: 15 m*M* Na_2CO_3, 35 m*M* $NaHCO_3$, pH 9.0.
8. Blocking buffer: PBS plus 2% BSA, 0.5% sodium azide.
9. PBS-Tween: PBS plus 0.05% Tween-20.
10. Horseradish peroxidase (HRP)-conjugated goat antimouse IgG (Jackson ImmunoResearch).
11. O-Phenylenediamine (OPD)-tablets (Sigma).
12. 30% hydrogen peroxide.
13. Sulfuric acid.
14. 0.1 *M* sodium citrate, pH 5.0.
15. ELISA Sealing Tape (Corning).

2.2. Receptor Transfectants as Immunogens

1. Transfected cell line expressing receptor of interest (*see* Chap. 10).
2. Parental cell line.
3. Appropriate growth and selection media for specific cell line.

3. Methods

3.1. Immunogens and Immunizations

3.1.1. Peptides

Peptides should be HPLC purified and conjugated to a carrier protein with some portion of the free peptide retained for screening. Both KLH and PPD carriers have been used to generate antichemokine receptor antibodies. Begin immunizations with 5–8 mice per immunogen.

1. Resuspend the conjugated peptide in PBS at 1 mg/mL and store the unused portion at –20°C until further use. DMSO may also be used if the peptide-conjugate is extremely hydrophobic and difficult to solublize in PBS.
2. Dilute 250 μL of conjugated peptide solution 1:2 in PBS.
3. Combine the 500 μL of conjugated peptide solution with 500 μL of either CFA (first immunization only) or IFA (subsequent immunizations, except final boost prior to fusion) and draw up into a 3-mL syringe. Attach a stopcock and a second empty 3-mL syringe and mix thoroughly by forcing the solution from one syringe into the other through the stopcock until a fine emulsion is produced. The emulsion may be tested by placing a small drop in a beaker of cold water. If the drop does not disperse within a few seconds, the emulsion is stable and ready for injection.
4. Transfer the emulsion to a 1-mL syringe and inject 200 μL per mouse intraperitoneally (ip) using a 25-gauge needle. Mice should be boosted at 10–14 d intervals with immunogen emulsified in IFA.
5. One week after the third or fourth boost, antisera may be collected and tested for immunoreactivity by ELISA using free peptide (*see* **Subheading 3.3.1.**) or by flow cytometry using receptor transfectants, cell lines or leukocytes known to express the receptor (*see* **Subheading 3.3.2.**).
6. Once immunoreactive sera is obtained, subsequent immunizations may continue ip or, a final boost with conjugated peptide in PBS may be given intravenously (iv) via tail-vein 3 d before fusion. Adjuvant is not used for the final iv boost.

3.1.2. Transfectants

For immunization with receptor transfectants, 10–20 × 10^6 cells per mouse are needed. If the transfectants were established in a syngeneic murine cell line, they must be treated with mitomycin C to prevent tumor formation.

1. Harvest the appropriate number of receptor transfectants and wash three times in PBS or growth media without FCS.
2. If mitomycin C treatment is necessary, resuspend the cell pellet in growth media without FCS at 5 × 10^7 cells/mL containing 25 μg/mL mitomycin C and incubate 30 min at 37°C.

3. Wash cells twice with PBS or growth media without FCS to remove the mitomycin C.
4. For immunization, resuspend the cells in PBS or growth media without FCS at 1×10^8 cells/mL. Draw cells into a 1–3 mL syringe using a 22-gage needle. Immunize mice ip with $10–20 \times 10^6$ cells (200 μL) using a 25-gage needle. Mice should be boosted at 10–14 d intervals following the same procedure.
5. One week after the third or fourth boost, antisera may be collected and tested by flow cytometry. Immunoreactivity of the antisera with receptor transfectants, however, does not correlate with immunoreactivity with the target receptor. This is because mice produce antibodies to many other cell surface determinants even when receptors are expressed in syngeneic cell lines. Depending on the transfectant host cell line, antisera may be better assessed by flow cytometry using transformed cell lines or leukocytes known to express the receptor or receptor transfectants made in a different cell line.
6. Once immunoreactive sera is obtained, subsequent immunizations may continue ip or, a final boost of cells may be given iv via tail-vein 3 d before fusion. A minimum of 4 boosts is recommended before fusion.

3.2. Fusion

$10–50 \times 10^7$ myeloma cells are needed per mouse spleen. Healthy myeloma cells are essential for a successful fusion. Cells should be expanded several days before fusion and kept in exponential growth phase ensuring that they do not over grow.

1. One day before fusion, myeloma cells should be split back to 2.5×10^5 cells/mL in DMEM plus 25 m*M* HEPES, 10% FCS, 1% sodium pyruvate.
2. Sacrifice mouse and aseptically remove the spleen to a 110-mm^2 tissue culture dish containing sterile serum-free DMEM without HEPES. All subsequent steps should be carried out in a tissue-culture hood.
3. Isolate spleen cells by flushing with two 3-mL syringes and 25-gauge needles.
4. Allow debris and clumps to settle to the bottom of a 15-mL conical tube and transfer the single-cell suspension to a 50-mL conical tube.
5. Pellet cells at 500*g*, 5 min, room temperature. After discarding the supernatant, resuspend the cell pellet in 5 mL of ammonium chloride solution to lyse red blood cells and incubate 5 min at room temperature.
6. Add 45 mL of serum-free DMEM, without HEPES and pellet cells.
7. Wash spleen cells and myeloma cells twice in serum-free DMEM without HEPES at room temperature and count cells.
8. Mix spleen cells and myeloma cells together in a 50-mL conical tube at a ratio of about 5 spleen cells/myeloma cell and pellet together at 500*g* for 5 min.
9. Discard supernatant and remove the last drops of media by aspiration. Resuspend the "dry" pellet by gently tapping.
10. Place the tube containing the cell pellet in a beaker of 37°C water. Keeping the tube at 37°C, add 2 mL of prewarmed (37°C) 50% PEG solution slowly over 1 min.

11. Gently mix by tapping and leave for 1 min.
12. Add 2 mL of warm serum-free DMEM without HEPES, *very slowly* over 1 min and gently mix.
13. Add 5 mL of warm serum-free DMEM without HEPES, *very slowly* over 1 min.
14. Add 25 mL of warm serum-free DMEM without HEPES and incubate for 10 min.
15. Pellet the cells at 500*g*, 5 min, no brake.
16. Resuspend pellet in 100 mL of prewarmed HAT media. (HAT media is DMEM plus 20% FCS, 1 m*M* sodium pyruvate, 1 m*M* L-glutamine, and 25 m*M* HEPES containing 1X HAT supplement.) Avoid vigorous pipeting.
17. Distribute 100 µL/well into 96-well flat-bottom plates (10 plates/fusion).
18. After 1 wk, feed cells by the addition of 100 mL HAT medium.
19. Culture supernatants will be ready to screen about 10–14 d after fusion.

3.3. Screening the Fusion

3.3.1. Peptide

MAbs produced by immunization with peptide may be screened first by ELISA to identify peptide specific antibodies. However, not all peptide specific antibodies will recognize the cell surface-expressed receptor. MAbs reactive with peptide must be subsequently screened by flow cytometry on receptor transfectants, cell lines, or leukocytes known to express the receptor in order to identify those which recognize native receptor. Alternatively, the fusion can be screened directly by flow cytometry to identify antibodies which recognize native receptor (*see* **Subheading 3.3.2.**).

To screen by ELISA:

1. Resuspend the unconjugated peptide set aside earlier at 1 mg/mL in PBS. DMSO may also be used if the peptide is extremely hydrophobic and difficult to solublize in PBS.
2. Dilute the peptide in carbonate buffer to 1–10 µg/mL.
3. Dispense into 96-well, NUNC-Immuno plates at 50 µL/well and incubate at 4°C overnight. Although 10 plates are required for the primary screen, additional plates will be needed for secondary screening and for screening limiting dilution clones.
4. Discard the peptide solution and add 300 µL of blocking buffer to each well and cover each plate with ELISA sealing tap or stack plates and wrap tightly with plastic wrap. Incubate at least 1 h at 37°C or overnight at 4°C. Plates may be stored at 4°C for several weeks in blocking buffer.
5. To screen hybridoma supernatants discard the blocking buffer and wash plates twice with 200 µL PBS-Tween per well.
6. Transfer 50 µL of hybridoma supernatant to the peptide coated plates and incubate 1 h at 37°C. Designate at least 1 well/screen and preferably 1 well/plate as a negative control. In place of hybridoma supernatant, add only DMEM plus 20% FCS to these wells.

7. Discard the supernatant and wash plates three times with 200 μL PBS-Tween/well.
8. Prepare 50 mL of enzyme-conjugated secondary antibody (goat antimouse-HRP) by diluting the stock 1:500 in PBS. Dispense 50 μL per well and incubate 1 h at 37°C.
9. Discard the secondary reagent and wash plate three times with PBS-Tween.
10. Prepare 50 mL of enzyme substrate by dissolving 1 OPD tablet into 12 mL of 0.1 *M* sodium citrate, pH 5.0, followed by the addition of 12 μL of water and 12 μL of 30% hydrogen peroxide. Dispense 50 μL/well and leave at room temperature for several minutes until a color change is evident.
11. Stop the reaction by addition of 25 μL of sulfuric acid (18 *M*) to each well.
12. Color change may be scored visually or by a plate reader using a wavelength of 490 n*M*.

3.3.2. Transfectants

MAbs produced by immunizing with receptor transfectants must be screened by flow cytometry—first against receptor transfectants to select for immunoreactive MAbs to the immunogen and then against the parental cell line to eliminate those immunoreactive antibodies recognizing determinants other than the transfected receptor. Mice will make antibodies to cell surface determinants other than the receptor of interest even when a syngeneic mouse cell line is used as the parental line for transfectants.

Screening by flow cytometry:

1. Harvest 5×10^7 receptor transfectant and wash once in ice-cold FACS buffer. Resuspend cells in 50 mL FACS buffer and dispense into 96-well V-bottom microtiter plates at 50 μL/well.
2. Transfer 50–100 μL of hybridoma supernatant to the wells and incubate at 4°C for 30 min. Dispensing the supernatants with moderate force will provide sufficient mixing. At this step it is important to designate one or two wells as negative controls to which hybridoma growth media only, not supernatant, will be added. All subsequent steps are the same for experimental and control wells.
3. Pellet cells by spinning plates at 500*g*, 3 min, 4°C. Aspirate off the supernatant taking care not to disturb the pellet and wash cells three times with 200 μL FACS buffer/well.
4. After the third wash, resuspend the cell pellet in 50 μL of FITC-conjugated secondary antibody (goat antimouse-FITC) prepared by diluting the antibody stock 1 : 500 in FACS buffer. Incubate for 1 h at 4°C.
5. Wash the cells three times, resuspending the final pellet in 300 μL of FACS buffer.
6. Analyze MAb immunoreactivity by FACS, setting parameters with the negative control first. It should be noted that brightness of staining is irrelevant at this point as it can be influenced by many factors, including receptor density and antibody concentration.
7. Immunoreactive supernatants from the primary screen should be immediately assessed on the parental cell line following the same procedure. In this secondary

screen, nonreactive supernatants are excellent candidates to follow for receptor specific MAbs. Supernatants immunoreactive with the parental line contain antibodies to cell surface determinants other than the receptor of interest. However, if time and resources permit these supernatants may be screened against leukocytes or receptor transfectants in a different cell background as they may also contain a receptor specific antibody obscured by antibodies to other determinants.

Receptor specific MAbs should be confirmed by staining cell lines or leukocytes known to express the receptor. Receptor transfectants made in a different cell background may also be used to confirm reactivity. Specificity of the MAbs may be evaluated by staining transfectants expressing different, but closely related receptors.

3.4. Limiting Dilution Cloning

Once supernatants containing immunoreactive antibodies have been identified in the primary screens, it is essential to clone the hybridoma as soon as possible for several reasons. The wells containing the hybridomas of interest are very likely to also contain nonantibody producing hybridomas, which generally grow faster than producers and/or hybridomas producing irrelevant MAbs. In addition, hybridomas are somewhat unstable particularly soon after they are generated and have a tendency to lose expression. Limiting dilution cloning ensures that the antibody is, in fact, monoclonal and allows for the selection of stable cell lines. Limiting dilution cloning may need to be performed two or more times with each hybridoma until all wells of a 96-well plate which are positive for growth are also immunoreactive with the antigen of interest AND the hybridoma has been diluted sufficiently that only 20% of the wells are positive for growth. Because the plating efficiency of hybridomas are variable and usually less than 100%, several dilutions may be necessary.

1. Resuspend the hybridoma in the 96-well plate and transfer to a 24-well plate contain 0.5 mL of HT media. The cells may be allowed to expand for 1 or 2 d but should be cloned as soon as possible.
2. Count cells and assess the viability for each hybridoma to be cloned.
3. Prepare three dilutions of each hybridoma such that 20 mL of HT media contains 100 cells (1 cell/well), 50 cells (0.5 cell/well), and 10 cells (0.1 cell/well).
4. Plate 200 μL/well of each dilution over one 96-well flat bottom tissue-culture plates.
5. After 7–10 d assess the number of wells positive for growth and assess the immunoreactivity of the supernatants.

References

1. Ponath, P. D. (1998) Chemokine receptor antagonists: novel therapeutics for inflammation and AIDS. *Exp. Opin. Invest. Drugs* **7,** 1–18.

2. Stadel, J. M., Wilson, S., and Bergsma, D. J. (1997) Orphan G protein-coupled receptors: a neglected opportunity for pioneer drug discovery. *Trends Pharm. Science* **18,** 430–437.
3. Qin, S., Rottman, J. B., Myers, P., Kassam, N., Weinblatt, M., Loetscher, M., et al. (1998) The chemokine receptors CXCR3 and CCR5 mark a subset of T cells associated with certain inflammatory reactions. *J. Clin. Invest.* **101,** 746–754.
4. Ponath, P. D., Qin, S., Post, T. W., Wang, J., Wu, L., Gerard, N. P., et al. (1996) Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. *J. Exp. Med.* **183,** 2437–2448.
5. Heath, H., Qin, S., Rao, P., Wu, L., LaRosa, G., Kassam, N., et al. (1997) Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. *J. Clin. Invest.* **99,** 178–184.
6. Ying, S., Robinson, D. S., Meng, Q., Rottman, J. B., Kennedy, R., Ringler, D. J. (1997) Enhanced expression of eotaxin and CCR3 mRNA and protein in atopic asthma. Association with airway hyperreactivity and predominant colocalization of eotaxin mRNA to bronchial epithelial and endothelial cells. *Eur. J. Immunol.* **27,** 3507–3516.
7. Loetscher, P., Seitz, M., Baggiolini, M., and Moser, B. (1996) Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. *J. Exp. Med.* **184,** 569–577.
8. Penton-Rol, G., Polentarutti, N., Luini, W., Borsatti, A., Mancinelli, R., Sica, A., et al. (1998) Selective inhibition of the chemokine receptor CCR2 in human monocytes by INF-γ. *J. Immunol.* **160,** 3869–3873.
9. Sallusto, F., Lenig, D., Mackay, C., and Lanzavecchia, A. (1998) Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J. Exp. Med.* **187,** 875–883.
10. Foxman, E. F., Campbell, J. J., and Butcher, E. C. (1997) Multistep navigation and the combinatorial control of leukocyte chemotaxis. *J. Cell Biol.* **139,** 1349–1360.
11. Jones, S. A., Wolf, M., Qin, S., Mackay, C. R., and Baggiolini, M. (1996) Different functions for the interleukin 8 receptors (IL-8R) of human neutrophil leukocytes: NADPH oxidase and phosolipase D are activated through IL-8R1 but not IL-8R2. *Proc. Natl. Acad. Sci. USA* **93,** 6682–6686.
12. Hammond, M. E. W., Lapointe, G. R., Feucht, P. H., Hilt, S., Gallegos, C. A., Gordon, C. A., et al. (1995) IL-8 induces neutrophil chemotaxis predominately via type IL-8 receptors. *J. Immunol.* **155,** 1428–1433.
13. Chuntharapai, A. and Kim, K. J. (1995) Regulation of the expression of IL-8 receptor A/B by IL-8: possible functions of each receptor. *J. Immunol.* **155,** 2587–2594.
14. Zagorski, J. and Wahl, S. M. (1997) Inhibition of acute peritoneal inflammation in rats by a cytokine induced neutrophil chemoattractant receptor antagonist. *J. Immunol.* **159,** 1059–1062.
15. Gong, J. H., Ratkay, L. G., Waterfield, J. D., and Clark-Lewis, I. (1997) An antagonist of monocyte chemoattractant protein 1 (MCP-1-inhibits arthritis in the MRL-*lpr* mouse model. *J. Exp. Med.* **186,** 131–137.
16. Lukacs, N. W., Strieter, R. M., Shaklee, C. L., Chensue, S. W., and Kunkel, S. L.

(1995) Macrophage inflammatory protein-1 alpha influences eosinophil recruitment in antigen-specific airway inflammation. *Eur. J. Immunol.* **25,** 245–251.
17. Broaddus, V. C., Boylan, A. M., Hoeffel, J. M., Kim, K. J., Sadick, M., Chuntharapai, A., and Hebert, C. A. (1994) Neutralization of IL-8 inhibits neutrophil influx in a rabbit model of endotoxin-induced pleurisy. *J. Immunol.* **152,** 2960–2967.
18. Rand, M. L., Warren, J. S., Mansour, M. K., Newman, W., and Ringler, D. J. (1996) Inhibition of T cell recruitment and cutaneous delayed-type hypersensitivity-induced inflammation with antibodies to monocyte chemoattractant protein-1. *Am. J. Path.* **148,** 855–864.
19. VanOtteren, G. M., Strieter, R. M., Kunkel, S. L., Paine, R. R., Greenberger, M. J., Danforth, J. M., et al. (1995) Compartmentalized expression of RANTES in a murine model of endotoxemia. *J. Immunol.* **154,** 1900–1908.
20. Harada, A., Sekido, N., Akahoshi, T., Wada, T., Mukaida, N., and Matsushima, K. (1994) Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J. Leukoc. Biol.* **5,** 559–564.
21. Baggiolini, M., Dewald, B., and Moser, B. (1997) Human chemokines: an update. *Ann. Rev. Immunol.* **15,** 675–705.
22. Weber, M., Uguccioni, M., Baggiolini, M., Clark Lewis, I., and Dahinden, C. A. (1996) Deletion of the NH2-terminal residue converts monocyte chemotactic protein 1 from an activator of basophil mediator release to an eosinophil chemoattractant. *J. Exp. Med.* **183,** 681–685.
23. Gong, J. H., Uguccioni, M., Dewald, B., Baggiolini, M., and Clark-Lewis, I. (1996) RANTES and MCP-3 antagonists bind multiple chemokine receptors. *J. Biol. Chem.* **271,** 10,521–10,527.
24. Koch, A. E., Polverini, P. J., Kunkel, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M., et al. (1992) Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* **258,** 1798–1801.
25. Strieter, R. M., Kunkel, S. L., Arenberg, D. A., Burdick, M. D., and Polverini, P. J. (1995) Interferon gamma-inducible protein 10 (IP-10), a member of the C-X-C chemokine family, is an inhibitor of angiogenesis. *Biochem. Biophys. Res. Commun.* **210,** 51–57.
26. Cao, Y., Chen, C., Weatherbee, J. A., Tsang, M., and Folkman, J. (1995) gro-beta, a-C-X-C-chemokine, is an angiogenesis inhibitor that suppresses the growth of Lewis lung carcinoma in mice. *J. Exp. Med.* **182,** 2069–2077.
27. Graham, G. J., Wright, E. G., Hewick, R., Wolpe, S. D., Wilkie, N. M., Donaldson, D., et al. (1990) Identification and characterization of an inhibitor of haemopoietic stem cell proliferation. *Nature* **344,** 442–444.
28. Nagasawa, T., Hirota, S., Tachibana, K., Takaura, N., Nishikawa, S., Kitamura, Y., et al. (1996) Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* **382,** 635–638.
29. Sarris, A. H., Broxmeyer, H. E., Wirthmueller, U., Karasavvas, N., Cooper, S., Lu, L., et al. (1993) Human interferon-inducible protein 10: expression and purification of recombinant protein demonstrate inhibition of early human hematopoietic progenitors. *J. Exp. Med.* **178,** 1127–1132.

30. Karpus, W. J. and Kennedy, K. J. (1997) MIP-1 alpha and MCP-1 differentially regulate acute and relapsing autoimmune encephalomyelitis as well as Th1/Th2 lymphocyte differentiation. *J. Leukoc. Biol.* **62,** 681–687.
31. Van Snick, J., Houssiau, F., Proost, P., Van Damme, J., and Renauld, J. C. (1996) I-309/T cell activation gene 3 chemokine protects murine T cell lymphomas against dexamethasone induced apoptosis. *J. Immunol.* **157,** 2570–2576.
32. Cook, D. N., Beck, M. A., Coffman, T. M., Kirby, S. L., Sheridan, J. F., Pragnell, I. B., and Smithies, O. (1995) Requirement of MIP-1α for an inflammatory response to viral infection. *Science* **269,** 1583–1585.
33. Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P. (1995) Identification of RANTES, MIP-1α, and MIP-1β as the major HIV-suppressive factors produced by CD8+ T cells. *Science* **270,** 1811–1815.
34. Xia, M., Qin, S., McNamara, M., Mackay, C., and Hyman, B. J. (1997) Interleukin-8 receptor B immunoreactivity in brain and neuritic plaques of Alzheimer's disease. *Am. J. Path.* **150,** 1267–1274.
35. Gupta, S. K., Lysko, P. G., Pillarisetti, K., Ohlstei, E., and Stadel, J. M. (1998) Chemokine receptors in human endothelial cells. Functional expression of CXCR4 and its transcriptional regulation by inflammatory cytokines. *J. Biol. Chem.* **273,** 4282–4287.
36. Chuntharapai, A., Lee, J., Burnier, J., Wood, W. I., Hebert, C., and Kim, K. J. (1994) Neutralizing monoclonal antibodies to human IL-8 receptor A map to the NH_2-terminal region of the receptor. *J. Immunol.* **152,** 1783–1789.
37. Chuntharapai, A., Lee, J., Hebert, C. A., and Kim, K. J. (1994) Monoclonal antibodies detect different distribution patterns of IL-8 receptor A and IL-8 receptor B on human peripheral blood leukocytes. *J. Immunol.* **153,** 5682–5688.
38. Endres, M. J., Clapham, P. R., Marsh, M., Ahuja, M., Turner, J. D., McKnight, A., Thomas, J. F., Stoebenau-Haggarty, B., Choe, S., Vance, P. J., Wells, T. N. C., Power, C. A., Sutterwala, S. S., Doms, R. W., Landau, N. R., and Hoxie, J. A. (1996) CD4-independent infection by HIV-2 is mediated by fusin/CXCR4. *Cell* **87,** 745–756.
39. Forster, R., Emrich, T., Kremmer, E., and Lipp, M. (1994) Expression of the G-protein-coupled receptor BLR1 defines mature, recirculating B cells and a subset of T-helper memory cells. *Blood* **84,** 830–840.
40. Frade, J. M. R., Llorente, M., Mellado, M., Alcami, J., Gutierrez-Ramos, J. C., Zaballos, A., del Real, G., and Martinez-A., C. (1997) The amino-terminal domain of the CCR2 chemokine receptor acts as a coreceptor for HIV-1 infection. *J. Clin. Invest.* **100,** 497–502.
41. Wu, L., Paxton, W. A., Kassam, N., Ruffing, N., Rottman, J. B., Sulliva, N., et al. (1997) CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. *J. Exp. Med* **185,** 1681–1691.
42. Wu, L., LaRosa, G., Kassa, N., Gordon, C. J., Heath, H., Ruffing, N., et al. (1997) Interaction of chemokine receptor CCR5 with its ligands: multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding. *J. Exp. Med.* **186,** 1373–1381.

28

Targeted Expression of Chemokines In Vivo

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1. Introduction

One of the main reasons for interest in chemokines is the ease with which their expression can be documented in physiological settings involving leukocyte trafficking or in diseases characterized by inflammatory cell infiltration. In the case of most ELR-containing CXC chemokines, their ability to attract neutrophils in vitro is paralleled by a similar activity when injected in vivo ***(1–3)***. However, in the case of non-ELR CXC and most CC chemokines, injection in vivo has resulted in disparate and inconsistent reports of activity ***(4–6)***. Thus, in order to be able to infer anything about the function of chemokines in normal physiology or disease, it must be demonstrated that their in vitro activities accurately predict their in vivo activities.

One way to address this issue is through the generation of genetically modified mice. Loss of function mutations engineered through targeted gene disruption allow investigators to identify function through altered physiology and assume that the absent chemokine is responsible for the absent activity ***(7,8)***. A complementary exercise is the use of gain of function mutations to test directly whether a chemokine's activity in vitro can be recapitulated in vivo ***(6,9–15)***. Furthermore, transgenic overexpression models permit organ-targeted expression of a chemokine thereby addressing other questions about leukocyte trafficking or inflammatory disease in specific organs. While genetic manipulation has the disadvantage of being labor intensive "up front," it has several advantages over direct in vivo administration of chemokines, including obviating the need for purchase of expensive proteins and surgical manipulation of the animal being analyzed (e.g., by installation of an osmotic pump to deliver the chemokine).

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In addition to facing standard problems inherent in constructing transgenic mice, the investigator undertaking these studies must address some variables that are unique to chemokines. For example, all transgenic work requires careful consideration of promoter choice, but with chemokines, an overly strong or widely expressed promoter can provide results that are nonphysiological and therefore difficult to interpret. In the case of monocyte chemoattractant protein-1 (MCP-1), a CC chemokine that attracts monocytes, memory T lymphocytes, and NK cells in vitro, transgenic expression under the control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) resulted in the appearance of transgenic MCP-1 in several organs, but no mononuclear cell infiltration ***(6)***. This was most likely due to the fact that, first, this promoter directed synthesis of enough MCP-1 that the mice had plasma levels of MCP-1 sufficient to desensitize their monocytes and, second, this promoter directs synthesis of MCP-1 in monocytes themselves, again potentially leading to receptor down-regulation. In contrast, when MCP-1 was expressed under the control of promoters that direct lower levels of expression in anatomically confined areas, monocyte infiltration was observed. For example, insulin promoter-driven MCP-1 transgenic mice suffered from a monocytic insulitis ***(15)***. There are several additional examples ***(11,12,14)***. This is not to say that high level systemic expression of a chemokine does not provide some information. In the case of the MMTV-MCP-1 mice, they were found to be more susceptible to intracellular pathogens (e.g., *L. monocytogenes* and *M. tuberculosis*), thereby implicating MCP-1 in host defense mechanisms against these bacteria. Nonetheless, these high plasma levels of MCP-1 are almost never observed in human disease. Rather, chemokines like MCP-1 naturally appear to exert their effects locally, and when expressed in this manner in transgenic models they are able to attract leukocytes in patterns that are predicted by their in vitro specificities.

Thus, in considering chemokine transgenic models, one should begin by framing an experiment that examines chemokine expression in a particular organ. The literature is replete with examples of organ-specific promoters that can be adapted for transgenic use. In constructing the transgene, the investigator should be aware that eukaryotic gene expression in chromatin is enhanced by the presence of introns. In our own experience, several transgenic lines constructed with a cDNA encoding MCP-1 resulted in no expression, while lines constructed with a genomic DNA clone provided high levels of expression with the same promoter. However, the introns need not be those indigenous to the gene being analyzed, since heterologous introns (e.g., for β-globin or human growth hormone) can accomplish the same task when positioned in the transgene in such a way that splicing will occur.

Once the transgene is constructed, it must be isolated free from as much

plasmid DNA sequence as possible since its presence tends to inhibit expression but not integration. Highly purified preparations of linearized transgene DNA are then injected into oocytes in vitro using a microinjection apparatus. Oocytes are then transferred surgically into the uteri of pseudopregnant female mice, which then act as foster mothers. Pups are analyzed for the presence of integrated copies of the transgene by extracting DNA from small pieces of surgically removed tail, and analyzing the DNA by Southern blotting or PCR. Mice that have integrated copies of the transgene are called "founder" mice. Founder mice and their offspring must then be analyzed to determine whether or not they express the transgene. RNA can be extracted from several organs and analyzed by Northern blotting or by RT-PCR if primers can be designed that span introns in order to exclude amplification of contaminating genomic DNA. Most desirable, however, is a demonstration of transgenic protein expression. This can be accomplished by immunoblotting or ELISA analysis of organ extracts if antibodies are available, or by measuring bioactivity of the transgenic protein. In this case, comparisons should be made to nontransgenic littermates, i.e., offspring of founder mice in outcrosses that have not inherited the transgene.

Once expressing founder lines are identified, they can be analyzed for physiological effects of the transgenic protein. Again, because of marked strain variability among mice (especially where inflammatory and immunological responses are concerned) it is important to make comparisons to nontransgenic mice of identical strain background. This can be accomplished either by using nontransgenic littermates of heterozygous mating pairs or, if the transgene has been bred to homozygosity, maintenance of a nontransgenic line of identical strain background.

This chapter will describe techniques for generating transgenic mice. Detailed descriptions of surgical techniques and setting up a microinjection apparatus are beyond the scope of this chapter. Instead, general guidelines will be provided along with references that provide a more complete picture (see **ref. *16***).

2. Materials

1. DNA isolation: Elutrap apparatus and Elutip columns (Schleicher and Schuell, Inc., Keene, NH). TAE: 40 m*M* Tris-acetate, pH 8.0, 1 m*M* EDTA. TE: 10 m*M* Tris, pH 7.4, 0.1 m*M* EDTA. Agarose gel electrophoresis boxes.
2. Microinjection (for complete descriptions, see Hogan et al. ***[16]***). Microinjector apparatus: Micromanipulators and instrument holders (Leitz or Narashige, Sea Cliff, NY); inverted microscope with Nomarski optics; Leitz capillary tubing; glass depression slides; 50-mL glass syringe; micrometer syringe. DeFonbrune microforge (Kramer Scientific Corp., Yonkers, NY); pipet puller.
3. Egg isolation and reimplantation:

a. Mice (Jackson Labs, Bar Harbor, ME). Pregnant mare serum (PMS; Organon Teknika Corp., Durham, NC). hCG (Sigma, St. Louis, MO).
b. M2 medium (modified Krebs-Ringer with HEPES): 94.66 m*M* NaCl, 4.78 m*M* KCl, 1.71 m*M* $CaCl_2$., 1.19 m*M* KH_2PO_4, 1.19 m*M* $MgSO_4$., 4.15 m*M* $NaHCO_3$, 20.85 m*M* HEPES, 23.28 m*M* sodium lactate, 0.33 m*M* sodium pyruvate, 5.56 m*M* glucose, 4 g/L BSA, 0.06 g/L penicillin, 0.05 g/L streptomycin sulfate, 0.01 g/L phenol red, pH to 7.4 with sodium hydroxide.
c. M16 medium (modified Krebs-Ringer with bicarbonate): 94.66 m*M* NaCl, 4.78 m*M* KCl, 1.71 m*M* $CaCl_2$., 1.19 m*M* KH_2PO_4, 1.19 m*M* $MgSO_4$., 25.0 m*M* $NaHCO_3$, 23.28 m*M* sodium lactate, 0.33 m*M* sodium pyruvate, 5.56 m*M* glucose, 4 g/L BSA, 0.06 g/L penicillin, 0.05 g/L streptomycin sulfate, 0.01 g/L phenol red, pH to 7.4 with sodium hydroxide.
d. Hyaluronidase (Sigma, St. Louis, MO).

3. Methods

3.1. DNA Preparation

1. Digest the plasmid containing the transgene with restriction enzymes that will separate the transgene from as much backbone plasmid DNA as possible. Separate the transgene DNA from the backbone plasmid DNA by agarose gel electrophoresis in the presence of ethidium bromide.
2. Visualize the transgene DNA using long wave UV and excise the band of interest.
3. Electroelute the DNA from the gel slice in TAE buffer.
4. Add 1/10 vol 3 *M* sodium acetate (pH 5.2) to the eluted DNA, and precipitate with 2 vol of ice-cold ethanol followed by centrifugation. Wash the pellet with ice-cold 70% ethanol.
5. Air-dry the pellet and resuspend in Elutip low salt buffer. Purify the DNA on Schleicher and Schuell Elutip-d column following the manufacturer's "Basic Protocol for DNA Purification."
6. To the eluted and purified DNA, add 1/10 vol 3 *M* sodium acetate, pH 5.2, precipitate with 2 vol of ice-cold ethanol, and wash the pellet with ice-cold 70% ethanol.
7. Air-dry the DNA and resuspend in sterile TE. Adjust vol to 100 ng/mL. (DNA will be diluted from this stock to the appropriate concentration on the day of injection.) Confirm the identity, purity, and concentration of the DNA fragment by agarose gel electrophoresis of 500 ng with standards of known concentration.

3.2. Isolation of Eggs

1. If mice are obtained commercially, allow them to adjust to their new light/dark schedule for 3–4 d. To induce super-ovulation, inject female mice 3–6 wk of age with 0.1 mL intraperitoneally (ip) of PMS (50 IU/mL) in normal saline between 1:00 and 2:00 PM (on a standard 5:00 am to 7:00 PM light cycle). Two days later, between noon and 1:00 PM, inject 0.1 mL of hCG (50 IU/mL) in NS ip. Place injected females in cage with stud male and check for plugs the following day.

2. Sacrifice a plugged female by CO_2 asphyxiation and mobilize the uterus, oviducts, and ovaries through a ventral midline incision. (Detailed surgical procedures are described in **ref. *16***.) Cut the oviduct near the ovary and cut the uterus below the oviduct. Transfer the oviduct with the small amount of attached uterus to a Petri dish containing a small amount of M2 culture medium.
3. Transfer 1 oviduct at a time to another Petri dish containing M2 supplemented with 300 μg/mL hyaluronidase and place under a dissecting microscope. Using forceps, tease the oviduct open to release eggs. Allow the hyaluronidase digestion to remove the cumulus cells, but as soon as they are gone, transfer the egg to a fresh dish of M2 medium without hyaluronidase.
4. Place 20–40 μL droplets of M16 medium in a 35 mm Petri dish in a two dimensional array, then add light paraffin oil to cover the droplets. Transfer eggs to these microdrop cultures and incubate at 37°C until ready for injection.

3.3. Injection of Eggs

1. To fashion a holding pipet, heat capillary tubing (1 mm OD) in a small flame and pull the ends apart. Score the thin area with a diamond pencil and break it. Examine the end under a dissecting microscope to make sure that the tip is flat. Place the pipet vertically in a microforge (or treat with a microflame) and observe the tip using an eyepiece with a micrometer. When the inside diameter is reduced to ≈15 μm, turn off the current. Then apply the heat from the microforge (or microflame) with the pipet in a horizontal position at a point 1–2 cm from the tip. Allow the pipet to bend to a 15° angle.
2. Use an automated pipet puller to make injection pipets from a 10–15 cm piece of glass tubing. The overall length of the pipets should be 5–8 cm. These should be prepared on the day of injection to reduce the likelihood of clogging.
3. Prepare the injection chamber by placing a drop of M2 medium in the center of the depression in a siliconized glass depression slide. Place it on the stage of an inverted microscope fitted with Nomarski optics.
4. Attach the holding pipet to a micromanipulator via its instrument holder, and connect it to a micrometer-driven syringe via Tygon tubing. Fill the syringe and holding pipet with light paraffin oil and place the tip in the injection chamber so that it lies horizontally and can be viewed through the microscope.
5. Transfer eggs to the chamber and view on high power to document the presence of pronuclei.
6. Dilute DNA solution to 1–2 μg/mL in sterile TE and fill the injection pipet by dipping the end in the diluted DNA solution. Allow capillary action to fill the pipet to several millimeters above the tip. Attach the injection pipet to a second micromanipulator via its instrument tube, and connect the instrument tube to a glass 50-mL syringe filled with air. Insert the injection pipet tip into the injection chamber at a 5–10° angle. Demonstrate that the injector is not clogged by displacing an egg with a stream of DNA solution.
7. Place the tip of the holding pipet next to an egg and immobilize it against the tip by applying negative pressure. Attempt to orient the egg so that the pronucleus is

aligned with the axis of the holding pipet and on the side of the egg away from the holding pipet tip.
8. Bring the injection tip next to the egg and flush a small amount of DNA solution out of the tip. Keeping the tip and the pronucleus in the same focal plane, insert the tip into the egg and continue into the pronucleus. Apply pressure to the glass syringe, looking for a slight swelling of the pronucleus. Withdraw the injection tip quickly. Continue to inject other eggs, replacing the injection pipet if it clogs or if eggs begin to lyse.

3.4. Embryo Transfer

1. Set up a mating between a 6 wk-old female Swiss outbred mouse and a vasectomized stud male. Vasectomized males are available commercially from Jackson Labs (Bar Harbor, ME) or vasectomies can be performed as described ***(16)***. Check for plugging the next day and use a plugged pseudopregnant female.
2. Use a pipet puller to prepare glass transfer pipets so that they are 2–3 cm long with a 120–180 μm diameter, and flame polish the end. Fill the pipet with a small amount of light paraffin oil, then an air bubble, then M2, followed by another air bubble. Fill the pipet with 5–7 eggs, followed by another air bubble and a small amount of M2.
3. Anesthetize the pseudopregnant female by intraperitoneal injection of a 2.5% Avertin solution at 0.02 mL/g. Using aseptic technique, make a transverse incision 1 cm to the left of midline just below the last rib. Manipulate the incision until it overlies the ovary or its fat pad which is visible through the peritoneal wall. Mobilize the peritoneum and make a small incision, then place a suture through one of the cut sides. Using blunt forceps, bring the fat pad through the incision, dragging the ovary, oviduct, and uterus along. Hold the fat pad outside the body by means of a clip.
4. Locate the infundibulum under a dissecting microscope, and create a hole in the bursa with fine forceps. Pick up the infundibulum and insert the transfer pipet into the ampulla. Using a mouth pipet, expel the two bubbles flanking the eggs into the uterus.
5. Reinsert the uterus, oviduct, ovary, and fat pad into the peritoneal cavity, appose the peritoneum with 1 or 2 sutures, then staple the skin wound.
6. Allow the mouse to recover on a heating pad.

3.5. Identification of Transgenic Mice

1. After weaning, littermates of the same sex can be housed together and distinguished by earpunch codes. Several coding systems have been described ***(16)***.
2. Anesthetize a mouse with Avertin as described in **step 3** of **Subheading 3.4.**, and use a scalpel to remove 1 cm of distal tail and place it in an Eppendorf tube.
3. The following protocol is based on Laird et al. ***(17)***. To each tail fragment, add 0.5 mL Tail Lysis Buffer (100 m*M* Tris HCl, pH 8.5, 5 m*M* EDTA, 0.2% SDS, 200 m*M* NaCl). Then add 5 μL of stock Proteinase K (10 mg/mL in H_2O) to each sample and seal the tubes well. Shake vigorously in a 55°C water bath overnight.

4. Vortex each sample, then spin briefly in a microcentrifuge to remove insoluble material. Prepare fresh tubes with 0.5 mL isopropanol and pour the clarified supernatant from each tail preparation into the isopropanol tubes. This should result in an immediately visible DNA precipitate.
5. Using a sterile toothpick or pipet tip, transfer the DNA precipitate to a clean tube. Remove any excess isopropanol that was transferred with the DNA and then add 0.5 mL TE. Redissolve the DNA by brief vortexing and, if necessary, heating in the 55°C water bath.
6. Extract the mixture twice with phenol:$CHCl_3$ and once with CHCl3. Add 1/10 vol 3 *M* Na acetate, pH 5.2, and precipitate with 2 vol of ethanol. Wash the precipitate with 70% ethanol and redissolve DNA in 100 μL TE. Determine DNA concentration by UV absorption.
7. Transgenes can be identified by Southern blot or PCR analysis.

3.6. Documenting Transgene Expression

1. RNA extraction: After humane euthanasia, organs are isolated and homogenized in 1 mL of 4 *M* guanidinium isothiocyanate, 25 m*M* sodium citrate (pH 7.0), and 100 m*M* 2-mercaptoethanol. RNA is purified from the extract by centrifugation through a cushion of 5.7 *M* CsCl followed by ethanol precipitation ***(18)***. Alternatively, RNA can be isolated using any of a variety of commercially available kits, e.g., from Qiagen (Chatsworth, CA). Transgene mRNA expression can then be determined by Northern blotting or RT-PCR.
2. Protein extraction: To isolate biologically active protein, organs can be placed in 1 mL cold 50 m*M* Tris-HCl, pH 7.5, 0.2 m*M* EDTA and homogenized on ice. Extracts are clarified by centrifugation at 15,000*g* for 30 min, followed by a second centrifugation at 100,000*g* for 45 min. This results in a preparation of protein which, in the case of chemokines, can be used for in vitro chemotaxis assays to determine bioactivity. The presence of transgenic protein in these preparations can also be documented by immunoblot.

4. Notes

1. DNA purity is critical for achieving reasonable integration frequencies. We have recommended Elutip purification, but other techniques are permissible, including glassmilk.
2. F1 or F2 hybrids or outbred mice tend to provide the largest number of fertilized eggs. Typical hybrids include C57Bl/6 X CBA, C57Bl/6 X DBA, C57Bl/6 X SJL, C57Bl/6 X Balb/c, or C57Bl/6 X C3H. In addition, however, inbred FVB/N mice have excellent reproductive performance as well as unusually large pronuclei (50–60% larger in volume than B6SJLF_1 pronuclei) ***(19)***. This facilitates microinjection of transgene DNA.
3. Make sure that stud males are caged alone for several weeks prior to caging with females to prevent suppression of testosterone by dominant males in the same cage, which lowers plugging efficiency.

4. Injections should be timed for the period when pronuclei are largest. Using a standard light/dark cycle with lights on from 5 AM to 7 PM, mating will usually take place at midnight (the midpoint of the dark period). The enlarging pronucleus will be at its maximal size before dissolution of the nuclear membrane between 2 PM and 7 PM on the day after mating.
5. Analyze multiple founders to exclude insertional mutagenesis as the cause of an observed phenotype.
6. Ideally, bioactivity due to transgenic protein should be confirmed by immunologically specific neutralization.
7. Breeding will be simplified by creating lines homozygous for the transgene. Although a doubling of the intensity of a signal on Southern blot may be an indication of homozygosity, measuring twofold differences in intensity can be unreliable. The only way to be certain of homozygosity is to perform two or more crosses to nontransgenic mice and document 100% transmission of the transgene.

References

1. Colditz, I., Zwahlen, R., Dewald, B., and Baggiolini, M. (1989) *In vivo* inflammatory activity of neutrophil-activating factor, a novel chemotactic peptide derived from human monocytes. *Am. J. Pathol.* **134,** 755–760.
2. Foster, S. J., Aked, D. M., Schroder, J. M., and Christophers, E. (1989) Acute inflammatory effects of a monocyte-derived neutrophil-activating peptide in rabbit skin. *Immunology* **67,** 181–183.
3. Larsen, C. G., Anderson, A. O., Oppenheim, J. J., and Matsushima, K. (1989) Production of interleukin 8 by human dermal fibroblasts and keratinocytesin response to interleukin 1 or tumor necrosis factor. *Immunology* **68,** 31–36.
4. Van Damme, J., Proost, P., Lenaerts, J. P., and Opdenakker, G. (1992) Structural and functional identification of two human, tumor-derived monocyte chemotactic proteins (MCP-2 and MCP-3) belonging to the chemokine family. *J. Exp. Med.* **176,** 59–65.
5. Zachariae, C. O. C., Anderson, A. O., Thompson, H. L., Appella, E., Mantovani, A., Oppenheim, J. J., and Matsushima, K. (1990) Properties of monocyte chemotactic and activating factor (MCAF) purified from a human fibrosarcoma cell line. *J. Exp. Med.* **171,** 2177–2182.
6. Rutledge, B. J., Rayburn, H., Rosenberg, R., North, R. J., Gladue, R. P., Corless, C. L., and Rollins, B. J. (1995) High level monocyte chemoattractant protein-1 expression in transgenic mice increases their susceptibility to intracellular pathogens. *J. Immunol.* **155,** 4838–4843.
7. Cook, D. N., Beck, M. A., Coffman, T. M., Kirby, S. L., Sheridan, J. F., Pragnell, I. B., and Smithies, O. (1995) Requirement of MIP-1 alpha for an inflammatory response to viral infection. *Science* **269,** 1583–1585.
8. Lu, B., Rutledge, B. J., Gu, L., Fiorillo, J., Lukacs, N. W., Kunkel, S. L., et al. (1998) Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J. Exp. Med.* **187,** 601–608.

9. Simonet, W. S., Hughes, T. M., Nguyen, H. Q., Trebasky, L. D., Danilenko, D. M., and Medlock, E. S. (1994) Long-term impairment of neutrophil migration in mice overexpressing human interleukin-8. *J. Clin. Invest.* **94,** 1310–1319.
10. Lira, S. A., Zalamea, P., Heinrich, J. N., Funetes, M. E., Carrasco, D., Lewin, A. C., et al. (1994) Expression of the chemokine N51/KC in the thymus and epidermis of transgenic mice results in a marked infiltration of a single class of inflammatory cells. *J. Exp. Med.* **180,** 2039–2048.
11. Fuentes, M. E., Durham, S., K., Swerdel, M. R., Lewin, A. C., Barton, D. S., Megill, J. R., et al. (1995) Controlled recruitment of monocytes/macrophages to specific organs through transgenic expression of MCP-1. *J. Immunol.* **155,** 5769–5776.
12. Nakamura, K., Williams, I. R., and Kupper, T. S. (1995) Keratinocyte-derived monocyte chemoattractant protein 1 (MCP-1): analysis in a transgenic model demonstrates MCP-1 can recruit dendritic and Langerhans cells to skin. *J. Invest. Dermatol.* **105,** 635–643.
13. Tani, M., Fuentes, M. E., Peterson, J. W., Trapp, B. D., Durham, S. K., Loy, J. K., et al. (1996) Neutrophil infiltration, glial reaction, and neurological disease in transgenic mice expressing the chemokine N51/KC in oligodendrocytes. *J. Clin. Invest.* **98,** 529–539.
14. Gunn, M. D., Nelken, N. A., Liao, X., and Williams, L. T. (1997) Monocyte chemoattractant protein-1 is sufficient for the chemotaxis of monocytes and lymphocytes in transgenic mice but requires an additional stimulus for inflammatory activation. *J. Immunol.* **158,** 376–383.
15. Grewal, I. S., Rutledge, B. J., Fiorillo, J. A., Gu, L., Gladue, R. P., Flavell, R. A., and Rollins, B. J. (1997) Transgenic monocyte chemoattractant protein-1 (MCP-1) in pancreatic islets produces monocyte-rich insulitis without diabetes: abrogation by a second transgene expressing systemic MCP-1. *J. Immunol.* **159,** 401–408.
16. Hogan, B., Costantini, F., and Lacy, E. (1986) *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
17. Laird, P. W., Zijderveld, A., Linders, K., Rudnicki, M. A., Jaenisch, R., and Berns, A. (1991) Simplified mammalian DNA isolation procedure. *Nucleic Acids Res.* **19,** 4293.
18. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18,** 5294–5299.
19. Taketo, M., Schroeder, A. C., Mobraaten, L. E., Gunning, K. B., Hanten, G., Fox, R. R., et al. (1991) FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proc. Natl. Acad. Sci. USA* **88,** 2065–2069.

29

Chemokine Knockout Mice

Marc E. Rothenberg

1. Introduction

As the chemokine family continues to grow in number, it is becoming increasingly important to determine the distinct function of each molecule. This is particularly important because of the apparent overlapping biological activity of some chemokines in vitro. One approach to determining the unique role of each chemokine is to genetically generate "knockout" mice by homologous recombination. Such mice are selectively deficient in the targeted gene and therefore the immunologic phenotype of these chemokine-deficient mice can be analyzed.

Modification of the mouse genome via transgenic technology is now routine ***(1)***. The mouse is an excellent experimental system for analyzing genetic mutations since the pattern of development and the number of genes found in humans and mice are similar. In addition, it is relatively inexpensive to house mice in comparison with other mammals. Furthermore, mice have a relatively short gestational period, a brief time to maturation and sexual maturity and large litter sizes. Techniques have been developed to integrate DNA in a site-specific manner by homologous recombination. Extensive manipulation of the mouse genome has been made possible by advances in the development of cell culture methods for growing, transfecting, and selecting homologous recombination in embryonic stem cells while maintaining these cells in a pluripotent undifferentiated state ***(2)***.

Embryonic stem cells are grown in culture and the manipulated DNA of interest is transferred by electroporation. The manipulated DNA contains markers which allow the cells that have undergone homologous recombination to be enriched. Following electroporation and selection, DNA is extracted from

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embryonic stem cells. Standard molecular biology techniques are used to identify clones which have undergone homologous recombination at one allele. Identified clones are then injected into blastocysts obtained from the uterus of pregnant mice. The injected blastocysts are then transplanted into the uterus of a pseudopregnant female where the embryo continues to develop. Offspring are referred to as chimeric since some of the animals are derived from the host blastocyst and some are derived from the injected embryonic stem cell which contains the mutated allele. If the manipulated stem cell contributes to the germ line, then the chimeras can be bred. Some offspring will be heterozygotes for the altered allele. This is referred to as germ-line transmission. The use of isogenic DNA (DNA from the identical strain of mice as the embryonic stem cell), the use of linearized DNA, and the use of two selectable markers improves the ratio of homologous to nonhomologous clones that survive the selection.

The most commonly used mouse strain for the derivation of embryonic stem cells is the 129 strain ***(3,4)***. This strain carries the agouti coat color locus which is dominant over the recessively inherited black coat color locus of the C57Bl/6 mouse strain from which the host blastocysts are often obtained. Offspring in which the 129 embryonic stem cells have contributed to the embryo will therefore have agouti and black fur due to the different origin of the cells. The agouti comes from the embryonic stem cells and black from the host blastocysts. If the embryonic stem cells have also contributed to the germ line, the mating of the chimera to a female with a recessive coat color results in offspring with the coat color of the embryonic stem cell-derived strain. Offspring that appear to be high degree chimera are screened for the presence of the mutated allele.

Gene disruption strategies have already elucidated the function of a few chemokines. The first chemokine knockout to be reported was the targeted disruption of the MIP-1α chemokine ***(5)***. Mice deficient in MIP-1α had a marked impairment in inflammatory responses following virus-induced myocarditis and pneumonitis. This knockout revealed an essential role for MIP-1α in the development of the inflammatory response following viral infection. The stromal cell derived factor-1 (SDF-1) gene was the second chemokine to be gene targeted in mice ***(6)***. This targeted disruption resulted in embryonic lethality due to the surprising essential role of SDF-1 in cardiac development. Immunologic analysis of embryonic blood revealed defects in hematopoiesis. These findings would not have been possible without the knockout analysis. Eotaxin is the third chemokine gene to have been deleted from mice ***(7)***. Eotaxin-deficient mice had an impairment in the early recruitment of eosinophils to the lung following allergen challenge. Additionally, they had a marked deficiency in the levels of tissue eosinophils indicating the critical importance

of eotaxin as a chemokine that regulates eosinophil homing to non-hematopoietic tissues. Lastly, MCP-1-deficient mice have a marked impairment in macrophage recruitment in response to peritoneal injection of thioglycolate ***(8)***. Additionally, they have an imbalance in Th1 versus Th2 cytokines. Similar knockout approaches will provide additional essential information regarding the complexity and importance of chemokines. Below is a brief outline of the method used to generate knockout mice. The reader is referred to more detailed resources ***(2–4)***.

2. Materials

2.1. Construction of Targeting Vector

1. Targeting vector: A vector containing a positive selection marker (e.g., neomycin-resistance gene), negative selection marker (thymidine kinase gene) and a multiple cloning site.
2. Embryonic stem cell: The embryonic stem cell line is typically derived from a male agouti 129/terSv embryo. The cell line must be maintained with meticulous culture procedures to retain normal karyotype and an undifferentiated state.
3. Irradiated embryonic fibroblast feeder cell layer to be used for growing the embryonic stem cell line: G418 resistant embryonic fibroblast cells are derived from embryos that carry a targeted disruption such as the β-2 microglobulin gene ***(9)***.
4. Leukemia inhibitory factor.
5. Electroporation apparatus and supplies.

2.2. Generation of Germ Line Chimeras

1. Super-ovulated C57Bl/6 females.
2. C57Bl/6 males.
3. Blastocysts derived from pregnant C57Bl/6 females at 3.5 d post-coitus.
4. Pseudo-pregnant Swiss Webster foster-mothers.
5. NIH Black Swiss females.
6. Dissection microscope with needles and appropriate manipulation devices for blastocysts.

3. Methods

3.1. Generation of Gene Targeting Construct

1. Genomic DNA flanking the region of the chemokine gene that will be deleted should be cloned into a targeting vector. Typically the 5' region of the endogenous gene should be around 5 kb and the 3' region of the gene should be around 3 kb.
2. The targeting vector should be carefully analyzed by restriction enzyme digestion, sequencing and hybridization patterns.
3. The vector is linearized with an enzyme that does not disrupt the targeting sequence.

4. The linearized vector is transfected into an appropriate embryonic stem cell by electroporation.
5. Approximately 10^5 transfected embryonic stem cells are selected. Transfected embryonic stem cells are grown on layers of γ-irradiated embryonic fibroblast cells. Transfected cells are plated on G-418 resistant embryonic fibroblasts feeder layers and selected in G-418 and after 36 h they are selected in Fialuridine (FIAU) or gancyclovir.
6. Double-resistant colonies are picked 8 to 10 d after selection. Each colony is dissociated with a small drop of trypsin/EDTA and transferred to the wells of a 24-well plate with continued G-418 selection. After 3 to 4 d, half of the cells in each well are frozen and the remainder used for isolation of DNA or reculture.
7. DNA is isolated from each embryonic stem cell.
8. Southern blot and PCR analysis is used to confirm appropriately targeted embryonic stem cells.

3.2. Generation of Germ Line Chimeras

1. Four-week-old C57Bl/6 females are super-ovulated with pregnant mare serum 2 d prior to mating and with human chorionic gonadotropin hormone on the day of mating with C57Bl/6 males.
2. Blastocysts are collected from pregnant C57Bl/6 mice 3.5 d post-coitus.
3. Approximately 15–18 embryonic stem cells are microinjected into each blastocyst.
4. Injected blastocysts are implanted into the uteri of pseudopregnant Swiss Webster foster-mothers and allowed to develop to term.
5. Male chimeras are identified based on the presence of agouti coat color.
6. These male chimeras are mated with NIH Black Swiss females. These animals are used for testing the germ line transmissions since they have large litters and their coat color is distinguishable from agouti. Germ line transmission is confirmed by agouti coat color in the F1 animals and by Southern analysis or PCR.
7. Heterozygote matings are established in order to generate homozygous deficient animals.

3.3. Phenotypic Analysis of Gene Targeted Mice

1. Embryogenesis: Determine if the gene targeting event results in embryonic lethality. This is accomplished by genotyping the offspring of heterozygote matings. The ratio of wild-type:heterozygote:knockout should 1:2:1 based on Mendelian principles of inheritance. If the gene deficiency has reduced the number of homozygotes, then the gene disruption has affected embryonic survival.
2. Hematopoiesis: Determine if the disruption of the gene has affected blood cell development. As an initial approach, the bone marrow cells are analyzed from both femur bones. Femurs are harvested and the bone marrow cavity is washed with saline and the retrieved leukocytes are counted and stained for differential analysis.
3. Immunologic analysis: Determine the peripheral blood cell counts and differentials. Additionally, lymphoid cells are analyzed for weight and cell number in the

spleen and thymus. Splenocytes and thymocytes are further characterized by fluorescent activated cell sorter analysis to determine differential lymphocyte counts.

4. Nonspecific inflammatory responses. Gross inflammatory responses to nonspecific triggers in the peritoneal cavity are assessed. Macrophage recruitment 48 h following the intraperitoneal injection of thioglycalate is assessed. Neutrophil response 18 h following the injection of sodium casein is determined.
5. Specific inflammatory responses: Leukocyte recruitment into the lungs is analyzed by broncho-alveolar lavage fluid counts following allergen challenge. Mice are subjected to standard protocols for induction of allergen-induced eosinophilic airway inflammation *(7)*. Typically mice are sensitized with intraperitoneal injection of ovalbumin and alum separated by 14 d. Ten d following the last dose of sensitization, mice are challenged with four daily doses of aerosolized ovalbumin. Eighteen and 48 h following the last dose of aerosolized ovalbumin, the mice are sacrificed and subjected to broncho-alveolar lavage. Additionally, the lungs are sectioned and stained with hematoxylin and eosin. Differential leukocyte counts in the lung parenchyma and broncho-alveolar lavage fluid are determined.

References

1. Wynshaw-Boris, A. (1996) Model mice and human disease. *Nature Genetics* **13,** 259–260.
2. Bronson, S. K. and Smithies, O. (1994) Altering mice by homologous recombination using embryonic stem cells. *J. Biol. Chem.* **269,** 27,155–27,158.
3. Joyner, A. L. (1993) *Gene Targeting: A Practical Approach*. Oxford University, Oxford, UK.
4. Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994) *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
5. Cook, D. N., Beck, M. A., Coffman, T. M., Kirby, S. L., Sheridan, J. F., Pragnell, I. B., and Smithies, O. (1995) Requirement of MIP-1 alpha for an inflammatory response to viral infection. *Science* **269,** 1583–1585.
6. Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., et al. (1996) Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* **382,** 635–638.
7. Rothenberg, M. E., MacLean, J. A., Pearlman, E., Luster, A. D., and Leder, P. (1997) Targeted disruption of the chemokine eotaxin partially reduces antigen induced tissue eosinophilia. *J. Exp. Med.* **185,** 785–790.
8. Lu, B., Rutledge, B. J., Gu, L., Fiorillo, J., Lukacs, N. W., Kunkel, S. L., et al. (1998) Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J. Exp. Med.* **187(4),** 601–608.
9. Zijlstra, M., Li, E., Sajjadi, F., Subramani, S., and Jaenisch, R. (1989) Germ-line transmission of a disrupted β_2-microglobulin gene produced by homologous recombination in embryonic stem cells. *Nature* **342,** 435–438.

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Chemokine Receptor Knockout Mice

Ji-Liang Gao and Philip M. Murphy

1. Introduction

Chemokines are a large family of secreted proteins that regulate the trafficking and activation of leukocytes by binding to G-protein-coupled receptors. In vitro studies of cloned and native receptors have shown that most chemokines bind to more than one receptor, and most chemokine receptors bind more than one chemokine. This suggests that the chemokine network may be a highly redundant system ***(1,2)***. Gene targeting has proven to be a useful tool for testing this hypothesis. So far the results suggest that each molecule has a specific biological role in vivo ***(3–12)***.

The ability to create gene-targeted mice is based on two phenomena. One is homologous recombination of DNA. As applied to knockout mice, this is the ability of a fragment of genomic DNA, when introduced into a mouse cell, to locate and recombine with the corresponding gene in its chromosomal context, a process also known as gene targeting ***(13)***. The second is the ability of embryonic stem (ES) cells to be cultured and manipulated in vitro relatively easily without losing totipotency or the ability to reinitiate the normal embryonic developmental program when placed back into embryos ***(14,15)***.

Two strategies, gene disruption and gene modification, have been used in gene targeting. Gene disruption, also known as gene knockout, is the most commonly used strategy. The goal of this technique is ablation of the function of the targeted gene by introducing a selectable marker gene ***(16)***. The goal of gene modification is to generate mutations without introducing selectable markers. In this chapter, we will only discuss gene disruption. Readers who are interested in gene modification are referred to references ***(17–21)***.

In general, gene knockout experiments include the following four steps. First, a targeting vector is created, in which a particular gene is disrupted and

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replaced by a positive selectable marker gene. The most commonly used gene for this purpose is *neo*, a bacterial gene encoding an aminoglycoside phosphoribosyltransferase which confers resistance to aminoglycosides such as G418 (geneticin). Second, mouse embryonic stem (ES) cells deficient in the targeted gene are generated by homologous DNA recombination. Third, the mutated ES cells are introduced into blastocysts of normal mice to generate chimeric embryos, which may develop into chimeric animals. Fourth, homozygous mice with targeted deletion of the gene are generated by several generations of breeding (**Fig. 1**).

2. Materials

2.1. Gene-Targeting Vector

Routine reagents and enzymes (depend on individual experiment) of molecular biology.

2.2. ES Cell Lines with Targeted Gene Deletion

1. Embryonic Stem Cell Media (ESM): DMEM with high glucose (Gibco, Cat. No. 12430), 15% fetal calf serum (FCS), 0.1 m*M* nonessential amino acids (100X stock, Gibco, Cat. No. 11140), 0.1 m*M* 2-mercaptoethanol (1,000X stock, Gibco, Cat. No. 21985), penicillin and streptomycin (100X stock, Gibco, Cat. No. 15140), and 1,000 U/mL leukemia inhibitory factor (LIF) (Gibco, Cat. No. 13275).
2. Embryonic Feeder Cell Media (EFM): DMEM with 10% FCS and 1X penicillin and streptomycin.
3. Hanks-HEPES (H/H) buffer: 500 mL of buffer is made of 438.75 mL H_2O, 50 mL of 10X Hanks buffer (Gibco, Cat. No. 14185); 10 mL of 1 *M* HEPES buffer (Gibco, Cat. No. 15630); and 1.25 mL of 1 *M* NaOH to adjust pH to 7.2.
4. Trypsin/EDTA solution: 0.25% trypsin and 1 m*M* EDTA (Gibco, Cat. No. 25200) for ES cell desegregation; 0.05% trypsin and 1 m*M* EDTA for EF cells.
5. ES cell freezing buffer (2X): 60% of ES media (ESM), 20% of FCS, and 20% of DMSO (Sigma, Cat. No. D5879).
6. Electroporation buffer: To make 50 mL of electroporation buffer, in 44 mL of distilled water add 1 mL of 1 *M* HEPES buffer, 0.1 mL of 2-mercaptoethanol, and 50 μL of 1 *M* NaOH.
7. Other reagents: Gelatin (Sigma-Aldrich, Cat. No. G2500), mitomycin C (Sigma-Aldrich, Cat. No. M0503), ganciclovir (Syntex), G418 (Gibco, Cat. No. 1181).
8. ES cell lysis buffer: 100 m*M* Tris-HCl, pH 8.5, 5 m*M* EDTA, 0.2% SDS, 200 m*M* NaCl, 100 μg/mL proteinase K (fresh added).
9. ES cell lines: Derivation of ES cells is beyond the scope of this article. ES cell lines are now available from ATCC. Readers interested in specific methods are referred to ***(14,15)***. Also, ES cell lines are now available from ATCC.

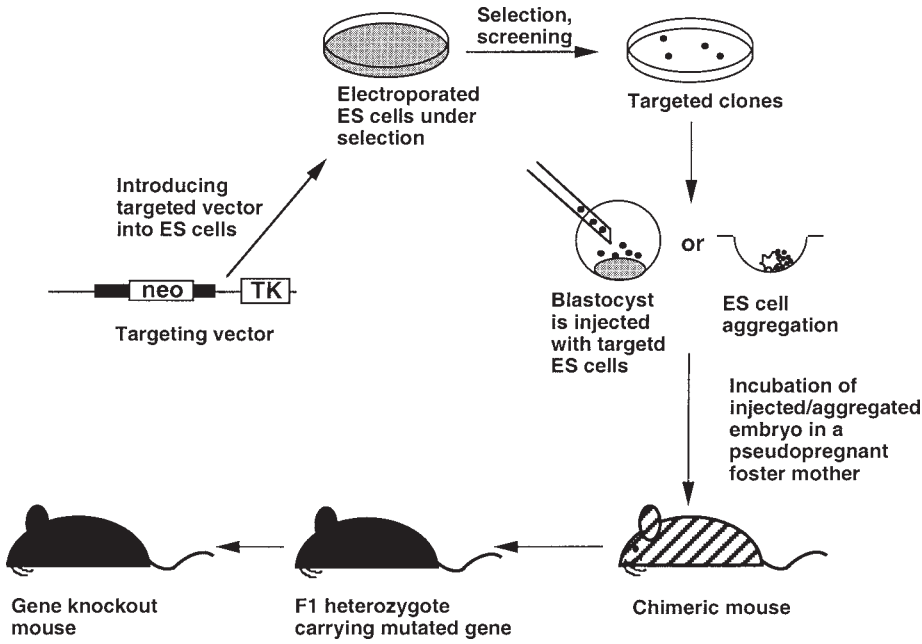

Fig. 1. Diagram of gene knockout experiment in mice. In a targeting vector, a particular gene is disrupted and replaced by a positive selectable marker gene (*neo)* and a negative selectable marker gene (TK). The targeting vector is introduced into ES cells by electroporation. The electroporated ES cells are selected with G418 and ganciclovir, and the surviving ES clones are screened by PCR or Southern blot to identify targeted ES cells. The targeted ES cells are introduced into blastocysts by microinjection or by aggregation. The blastocysts with targeted ES cells are then transferred into a pseudopregnant foster mother, and chimeric mice are generated. The chimeric mice are bred with wild-type mice to generate heterozygotes carrying the mutated gene. The gene knockout mice are obtained by breeding heterozygotes.

2.3. Chimeric Mice and Knockout Mice

1. Animal facility and equipment: a specific pathogen free (SPF) Animal facility for mice is required; other equipment is indicated where needed in the methods section.
2. Mouse tissue (mouse tail) lysis buffer (2X): 8 *M* urea, 20 m*M* EDTA (prepared from a stock solution of 0.5 *M*, pH 8.0, 1% N-lauroylsarcosine (Sigma-Aldrich, Cat. No. L-9150) (make stock solution, 30%), 0.2 *M* Tris-Cl, and 0.4 *M* NaCl. The 2X lysis buffer is diluted to 1X with distilled water. Proteinase K is added to 100 μg/mL when needed.

3. Methods

3.1. Construction of the Gene-Targeting Vector

The design of a gene targeting vector is the first and the most important step of a gene targeting experiment since it will determine the progress of the rest of the experiments, not only in the generation of the mutated allele, but also in the functional analysis of resultant animals. The targeting vector used for gene disruption is a replacement vector, also called Ω-type vector ***(22)***, which consists of three main elements, homologous sequences of the targeting gene, a positive selectable marker, and bacterial plasmid sequences. In most cases, a negative selectable marker is used to enrich the ES cells with homologous recombination. The primary parameters to be considered in the design of a replacement vector are the homologous sequences, the location of the positive selectable marker, and the method used for the screening. The following are guidelines for creating a replacement vector that will generate an easily identifiable null allele.

1. A gene fragment used to construct a targeting vector should be cloned from the same mouse strain as the ES cells to reduce the effect of polymorphic variation, since polymorphic variation between the vector and the chromosome of the ES cell may significantly decrease the targeting frequency.
2. The length of the homologous sequences of the vector should be five to eight kilobases. Longer homology may help the targeting frequency, but is more difficult to manipulate. The open reading frames (ORFs) of most chemokine receptor genes reside on a single exon with size of about 1.1 kb ***(23)***, thus, the size of the gene fragment should be about 6–9 kb (most of the 1 kb ORF will be deleted). Because of the insertion of the positive selectable marker, the homologous sequences are divided into two arms. Equal length of two arms may increase the targeting frequency, but for polymerase chain reaction (PCR) screening, a fragment larger than 2 kb is difficult to be amplified, so the homologous sequences are divided into a long arm and a short arm.
3. The ORF should be deleted as much as possible. However, large deletions including regions outside the ORF may affect other genes since genes for many chemokine receptors are very tightly linked ***(24)***. If only part of the ORF is deleted, the positive selectable marker should be inserted at the 5' end to disrupt the coding sequence as much as possible. Partial deletion may generate a truncated protein which may have some biological function (although so far there is no evidence that truncated G-protein-coupled receptors maintain biological function in knockout mice).
4. An easy and effective screening strategy has to be considered when designing a knockout vector. Two methods have been successfully used, PCR and Southern blot analysis. The advantage of PCR is that a large number of samples can be screened in a short time. For experiments with very low targeting frequency,

PCR screening might be an option because samples can be pooled (normally five) in primary screening. Once a positive pool is identified, targeted clones can be confirmed by Southern blot. The disadvantage of PCR screening is that it takes time to optimize the PCR conditions, and sometimes, positive clones will be missed. For most of the knockout experiments with positive and negative selection, the targeting frequency is 1/10 to 1/100; therefore, Southern blot is the most reliable and straightforward method. Following is a brief discussion of the two screening methods.

a. PCR-based screening: Primers should be designed in such a way that one of the primers can anneal to the outside region of the targeting vector, and the other can anneal to the positive selectable marker, so that a specific junction fragment between chromosome and the vector can be amplified and the homologous recombinants can be screened. Because the efficiency of PCR amplification is related to the size of the product, the distance between the two primers cannot be too far. On the other hand, the size of the PCR product cannot be less than the length of the short arm of the homologous sequences. Considering both PCR requirements and targeting efficiency, the short arm is usually around 1.5–2 kb. Successful targeting with 500 base pairs has been reported, but the frequency was low.
b. Southern-blot based screening: To increase the targeting frequency, the homologous sequences should be evenly distributed on both sides of the positive selectable marker. To distinguish homologous recombinants from nonhomologous recombinants, the restriction enzyme sites and the probe should be at the outside of the vector, and the resultant band should be easily distinguishable from the wild type. Using a CCR1 knockout vector as an example (**Fig. 2**), two *Xba*I sites and the probe are in the outside of the targeting vector, and the mutated band is about 1 kb larger than wild-type, which is easily distinguishable. If the restriction enzyme site also exists in the vector, two external probes have to be used to check the fidelity of the homologous recombination in both sides of the targeted gene.

5. Using combined positive-negative selection to enrich for ES cells with homologous recombination. The HSV-TK (herpes simplex virus thymidine kinase) gene is commonly used as the negative selectable marker in the targeting vector (**Fig. 2**). After transfection, the ES cells will be selected with both ganciclovir and G418. Most cells that have random insertion of the vector will express the TK gene, and will be killed by ganciclovir which is activated by specific phosphorylation by HSV-TK. Cells lacking HSV-TK will be resistant to ganciclovir. Using this strategy, targeted colonies can be enriched by 3–30-fold.
6. Plasmids used for knockout vector. The plasmid pPNT is widely used for gene knockout experiments. This vector contains *neo* and HSV-TK ***(25)***. The two arms of the homologous sequences can be inserted into two cloning sites flanking *neo*. In case the cloning sites do not exist in the homologous sequences, PCR-amplified products can be used, in which appropriate sites are created.

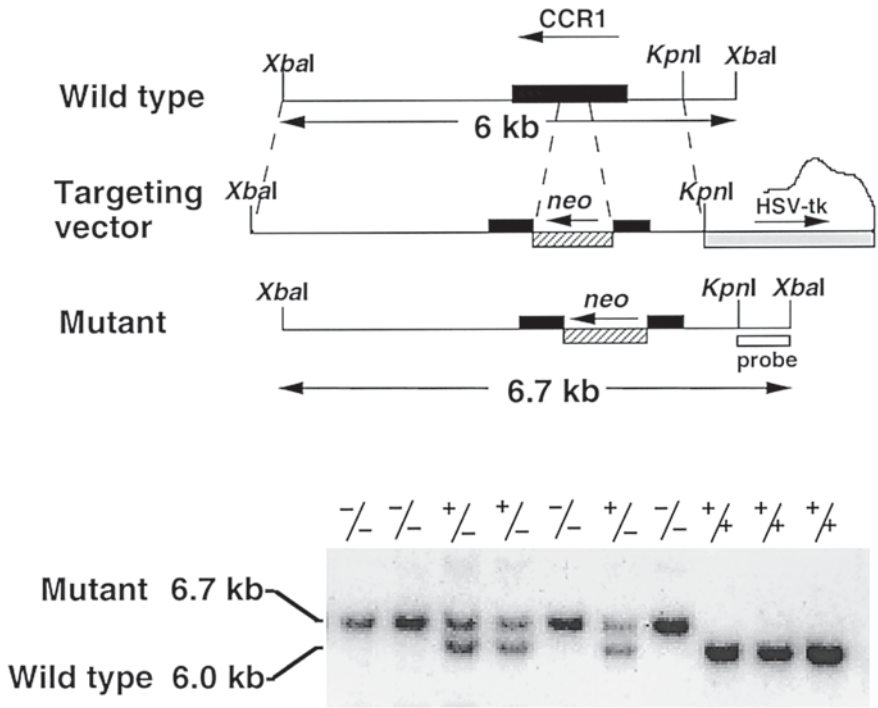

Fig. 2. Mutagenesis strategy. A *neo* gene replaced one-third of the ORF and was used as a positive selectable marker. The HSV thymidine gene (HSV-*tk*) was used for negative selection. The vector was linearized with *Xba*I digestion. The targeted events were screened by Southern blot. Two *Xba*I sites are in the outside of the targeting vector (the 3'-end one is just at the end of the vector, which cannot be digested by *Xba*I if the vector is randomly inserted). An outside probe is located at the 5'-end. The genotype of the mice can be easily identified based on the size of the hybridized bands, 6.7 kb (mutant) vs 6 kb (wild-type).

3.2. Creating ES Cell Lines with Targeted Gene Disruption

Keeping ES cells undifferentiated is a key to success in achieving germ line transmission of the targeted allele. To prevent differentiation, ES cells must grow either on monolayers of mitotically inactivated fibroblast cells (embryonic feeder [EF] cells) or in the presence of leukemia inhibitory factor (LIF). In our experiments, we use both feeder layer cells and LIF-supplemented media for ES cell culture and maintenance.

3.2.1. Preparation of Embryonic Feeder (EF) Cells

The EF cells are prepared from 14–16-d-old embryos (from 14–16 d post-coitus pregnant mice). Since most of the EF cells used for ES cells are under

G418 selection, transgenic mice that express *neo* should be used. A large stock of EF cells can be prepared and frozen. The stock EF cells can be thawed when needed. For each preparation, 7–10 embryos are usually used.

3.2.1.1. Preparation of Stock EF Cells.

1. Sacrifice a pregnant mouse (about 15 d postcoitus), moisten the belly with 70% ethanol, remove the uterus and place it in a 10-mm Petri dish containing phosphate-buffered saline (PBS). Dissect the embryos away from the uterus and membranes, and transfer them into a new dish containing PBS.
2. Remove the liver and heart, and wash twice with PBS to remove as much blood as possible.
3. Mince the carcasses in PBS and sieve out the blood with a cell strainer (Becton Dickinson, cat. no. 2360).
4. Transfer the minced carcasses into a sterile flask. With a stir bar, add 50 mL of 0.05% trypsin/EDTA solution, stir for 30 min, and pipet several times every 10 min.
5. If the solution becomes too viscous due to released DNA, add 200 μL of DNAse I (10 mg/mL).
6. Add another 50 mL of trypsin/EDTA solution and stir for another 30 min.
7. Centrifuge the cells at 270*g* for 5 min and resuspend the cell pellet in 10 mL EF media.
8. Count the cells, and plate 5×10^5 in each 10-cm tissue culture dish with 10 mL EF media. Incubate in a 37°C incubator with 5% CO_2.
9. Change media after 24 h to remove cell debris.
10. When the plates are confluent (2–7 d depending on preparation), trypsinize the cells and expand to five plates for each original plate of cells.
11. When the plates are confluent again (2–3 d), harvest the cells, and freeze the cells with EF freezing media (10% DMSO in EF media). Normally, we freeze 5×10^6 cells/freezing vial with 1 mL of EF freezing media.
12. Place the freezing ampoules inside a polystyrene box, seal the box, place in a –70°C freezer overnight. Then transfer the ampoules to liquid nitrogen storage after 24 h.

3.2.1.2. Preparation of EF Cells for ES-Cell Culture

One week before the date planned for electroporation of the targeting vector two vials of *neo*-EF cells should be thawed and expanded to eight 150-cm^2 flasks, treated with mitomycin C, and plated in 6-cm pregelatinized plates for ES-cell culture after electroporation. (Plates are gelatinized by incubating each plate with 4 mL sterile 0.2% gelatin solution for 1 h at room temperature, and suck out gelatin solution before loading cells.)

1. Thawing EF cells: EF cells are thawed quickly in a 37°C-water bath. Each vial of cells is transferred into 10 mL of EF media and centrifuged (270*g*, 5 min). The cell pellet is resuspended in 10 mL of EF medium and cultured in a 75-cm^2 flask.

2. Expansion of EF cells: Each flask of cells is expanded into two 75-cm^2 flasks after 2 d. After two more days, two flasks of the cells are expended into four 150–cm^2 flasks.
3. Mitomycin C treatment of EF cells: Two days later, replace EF media with 20 mL of mitomycin C media (1% mitomycin C in EF media), incubate for 3 h, carefully remove the media, and wash the cells once with PBS. (An alternative way is gamma radiation. The cells are trypsinized, rinsed, resuspended in the media, and exposed to 6,000–10,000 rads of gamma irradiation.)
4. Harvesting of EF cells: Trypsinize the cells with 3 mL of 0.05% trypsin-EDTA solution for about 1 min at 37°C, add 5 mL of EF media to neutralize trypsin, and pellet the cells by centrifugation. At this point, EF cells can be directly used for ES-cell culture or can be frozen for later use (5×10^6 cell per vial).
5. Plating of EF cells for coculture with ES-cells: Resuspend the cell pellet in EF media, count cells and make final dilution to 5×10^5 cells per 5 mL, and place 5 mL of the cells into each gelatinized 6-cm plate. About 65 plates are usually obtained from two vials of stock EF cells. The cells will be ready in 12 h and good for 10 d. Media should be changed every 3 d.

3.2.2. ES-Cell Culture

Six days before the electroporation date, one vial of frozen ES cells should be thawed, and expanded to four 6-cm plates. This should give 2×10^7 cells for electroporation.

1. Before thawing ES cells: One day before thawing ES cells, thaw one vial of mitomycin C-treated EF cells (~5×10^6 cells), and plate in 6–10 pregelatinized 6-cm plates.
2. Thawing ES cells: Frozen ES cells are thawed quickly in a 37°C-water bath. The cells are transferred into 10 mL of ES media and centrifuged (270*g*, 5 min). The cell pellet is resuspended in 5 mL of ES media and dispensed into a 6-cm plate with EF cells, and the media is changed daily. The cells should be split before confluence, usually at day 3.
3. Sub-culturing ES cells: Refeed ES cells 2–3 h prior to subculturing. Wash ES cells with H/H buffer, add 1 mL 0.25% trypsin-EDTA, and incubate for 2.5 min in a 37°C incubator. Shake the plate, incubate for another 2.5 min, gently pipet the cells up and down several times to break cell clumps, add 5 mL ES media and pipet up and down for another several times, centrifuge for 5 min at 270*g*, resuspend the cells in ES media and determine the cell concentration. Pass 5×10^5 cells to a new 6-cm EF-cell plate.
4. ES cells for electroporation: Go to **Subheading 3.2.3.**
5. ES cells for stock: If the ES cells are not used for electroporation, they can be frozen. Harvest the cells by trypsinization as described in **step 3** of **Subheading 3.2.2.**, count the cells and resuspend to 2×10^6 cells/mL, dispense 0.5 mL of cells to a freezing vial with 0.5 mL 2X ES freezing media. Place the freezing vials

inside a polystyrene box, seal the box and place overnight in a –70°C freezer, and transfer the vials to liquid nitrogen storage after 24 h.

3.2.3. Electroporation and Selection of ES-Cells

1. DNA preparation: Linearize 25 μg of vector DNA with appropriate restriction enzyme, precipitate the linearized DNA, wash twice with 70% ethanol, dry the DNA, and redissolve in electroporation buffer at 1 μg/μL, centrifuge 30 min, transfer supernatant to an ethanol-cleaned tube, incubate the DNA at 42°C for 1 min, and place on ice.
2. ES cell preparation: Trypsinize subconfluent ES cells to a single cell suspension as described in **Subheading 3.2.2.**, centrifuge the cells, resuspend the pellet in 10-mL electroporation buffer, determine the cell density, centrifuge the cells again and resuspend the pellet with electroporation buffer to 2×10^7 cells/mL.
3. ES cell electroporation: Transfer 20 μL DNA into a clean 0.4 cm electrode cuvet (Bio-Rad) add 0.8 mL of ES cells, mix ES cells and DNA, and electroporate using a Bio-Rad Gene Pulser set at 0.4 kV and 25 μF (the time constant should be 0.4 to 0.5 s). Set the electroporated cells at room temperature for about 10 min.
4. ES cell culture: Resuspend the electroporated cells with ES media to 2.5×10^5 cells/4 mL, and add 4 mL of the ES cells to each 6-cm EF plate. Two plates are labeled with single selection (G418), and all the others with double selection (G418 + ganciclovir). Also, set up one plate as a control in which ES cells have not been electroporated.
5. ES cell selection: 24 h later start the single and double selection by replacing the ES media with media containing either G418 or G418 + ganciclovir. Replace with G418 ES media in the control plate. Control cells will die in a few days. Change the media daily, and stop double selection at day 5, and replace the media with G418-ESM (cells grow faster without ganciclovir). Pick colonies 6–12 d after selection, usually at day 7 after selection.

3.2.4. Picking, Harvesting, and Freezing ES-Cell Colonies

3.2.4.1. Preparations

1. At the day of electroporation, thaw two vials of *neo*-EF cells, and expand to eight 150-cm^2 flasks and treated with mitomycin C. The EF cells are then plated in pregelatinized 24-well plates (10^5 cells per well). Change the media G418-ESM 4 h before cell harvesting.
2. Set up a 25X dark-field dissecting scope in the hood.
3. Add 30 μL of 0.25% trypsin/EDTA to each well of a 96-well U-bottom plate.

3.2.4.2. Picking ES Colonies

1. Aspirate the media from the plates with ES colonies and replace with H/H buffer.
2. Using a dark-field dissecting microscope, aspirate one colony into the capillary tube by mouth suction and transfer it into one of the wells of a 96-well U-bottom plate. Collect 16 colonies (i.e., 2 vertical rows) every time.

3. Incubate at 37°C for 5 min, agitate with yellow tips 2–3 times using a multi-channel-multidispenser (load with yellow tips every second slot, 4 tips each time). Do not try to make a single cell suspension, check that colony has dispersed by naked eye.
4. Transfer 50 μL of G418-ES media from the 24-well EF-cell plate into each well and agitate aggressively 10 times.
5. Dispense the cells into a 24-well EF-plate, and put into 37°C incubator.
6. Change media with G418-ESM every day.

3.2.4.3. Harvesting and Freezing ES Colonies

1. Harvest the ES cells at about 50% confluence (~2–3 d).
2. Aspirate the entire 24-well plate and wash once with H/H buffer.
3. Add 200 μL of 0.25% trypsin/EDTA to each well.
4. Incubate at 37°C for 5 min.
5. Add 400 μL ESM to each well and agitate 10 times with a 1-mL tip.
6. Dispense 400 μL cell suspension into a freezing ampoule with 400 μL of 2X freezing media, and mix.
7. Dispense the rest of the cell suspension into a new pregelatinized 24-well plate with 0.5 mL ESM for DNA preparation after 4–5 d. Make sure that the labeled numbers match with the numbers on freezing ampoule.
8. Place the freezing vials inside a polystyrene box, seal the box and place in a –70°C freezer overnight, and transfer the vials to liquid nitrogen storage after 24 h.

3.2.5. Screening ES Colonies for Homologous Targeting Events

3.2.5.1. DNA Isolation from ES Cells

1. When the ES cells in 24-well plates are confluent, aspirate the medium, and wash with PBS once.
2. Add 500 μL of ES-cell lysis buffer to each well, and incubate for 3 h or overnight at 37°C.
3. Add 500 μL of isopropanol to each well, shake for about 15 min on an orbital shaker until a DNA precipitate is visible.
4. Fish out the precipitated DNA with a yellow tip, and transfer the DNA into an Eppendorf tube with 500 μL of 70% ethanol.
5. Spin 1 min, discard ethanol, add 500 μL TE buffer to each tube, and dissolve DNA by incubatation at 60°C for 3 h, or at 37°C overnight.

3.2.5.2. Analysis of ES Cell DNA by Southern Blot or PCR

Based on the screening strategies discussed in **Subheading 3.1.**, targeted colonies can be screened by PCR or Southern blot. Take 15 μL DNA for each restriction enzyme digestion, or, pool 5 samples of DNA to run PCR. The amount of DNA in each PCR reaction should be optimized with other reagents.

3.3. Creating Chimeric Mice from the Targeted ES Cells

Mammalian embryos in the early stages are able to functionally incorporate cells from other embryos. Using this property, the mutated ES cells are introduced into embryos to create chimeric mice, in which some tissues are derived from the ES cells. If the germ cells in the chimeric mice are derived from the targeted ES cells, the targeted gene may be passed onto the next generation (F1), and gene-knockout mice could then be created by mating heterozygotes.

Currently, two methods are used to introduce ES cells into embryos, microinjection and aggregation. The microinjection method is the most commonly used. Its advantage is that it gives reproducible results, but it requires expensive equipment and good training. For a laboratory whose transgenic needs are episodic, it may not be worth setting up expensive and complicated equipment. Moreover, transgenic core facilities are now in place in many institutions for collaboration or service. Detailed equipment requirements and procedures can be found in **ref. *26***, and will not be discussed in this chapter.

The ES cell aggregation method, on the other hand, is a relatively easy technique, and does not need expensive equipment or extensive experience, but demands higher quality ES cells (early passage cells are required). This method was developed by Nagy et al. ***(27)***. After microinjection or aggregation, blastocysts are transferred into the uterus of pseudopregnant mice using standard procedures ***(26)***.

In order to identify chimeric mice easily, the recipient embryos are chosen to be from a strain that has a coat color different from the one from which the ES cells were derived. Most ES cell lines have been derived from Sv/129 mice which have agouti coat color. For microinjection, recipient embryos are usually from C57BL/6 because of its black color and ready availability from commercial vendors. For ES cell aggregation, CD1 (with white color) embryos were successfully used as recipient embryos originally in Nagy's group, and later in other groups including ours. In general, the degree of coat color chimerism is correlated with the degree of germline contribution. If the ES cell contribution in a chimeric mouse is more than 50% in its coat color, the probability of transmitting the germline into offspring is about the same.

3.4. Knockout Mice

Knockout mice can be produced by breeding chimeric mice. In addition to the time required to produce the targeted ES cell clone, the minimum time between the introduction of ES cells into embryos and the birth of –/– mice is five months. A breeding program has to be well planned to avoid any mistakes which may increase the time considerably.

3.4.1. Obtaining and Maintaining the Targeted Allele

Once chimeric mice are obtained, the first priority is to obtain and maintain the targeted allele in living animals. Not every chimeric mouse has the ability to transmit the mutated allele to subsequent generations. It is obvious that dominant lethal mutations cannot be maintained at all if the lethality occurs before sexual maturity or if it effects the gamete function. In this case, the chimera itself is the only transmitter of the mutated allele. Otherwise, the best and most economical way to identify a germline chimera and to get germline transmission is by breeding the chimeras with wild-type mice. To increase the chances of germline transmission, the chimeric mice and backcrossed strain have to be well selected. The chimeras should be male mice with high degree of ES cell contribution. There are two reasons to choose male chimeras. First, male chimeras can be bred more rapidly since they can mate with multiple females. Second, since all of the ES cell lines in common use were derived from male embryos, and XY cells do not normally undergo oogenesis in chimeras, the chance of germline transmission for female chimeras is very low, unless part or all of the Y chromosome is lost, resulting in effective XO cells which are capable of forming ova. In these cases, the produced offspring may have genetic defects.

The mice used for breeding with chimeras should have genetic markers which will allow discrimination of offspring derived from ES cells or derived from the host embryos. For example, chimeras derived from Sv/129 ES cells and C57BL/6 embryo are usually bred with C57BL/6, and their offspring can be easily distinguished based on their coat color: agouti animals are derived from Sv/129 ES cells (half of them will carry the targeted allele in most cases) and the black animals are derived from host embryos. The mice used for breeding with chimeras should also have high reproductivity which is helpful to get germline transmission faster. C57BL/6 mice have this property.

3.4.2. Identification of the Mutated Allele by Mouse DNA Analysis

The targeted allele can be identified by DNA analysis using PCR. The following procedure can be used to isolate mouse DNA for identifying the targeted allele.

1. A small piece of mouse tissue (usually a piece of mouse tail about 0.5 cm long, or a small piece of mouse ear) is digested in an Eppendorf tube with 0.5 mL of mouse tail lysis buffer overnight at 55°C.
2. Microcentrifuge for 3 min, carefully pour the supernatant into a new Eppendorf tube with 750 μL of ethanol, and mix by inverting the tube several times. The precipitated DNA will appear as cotton-like threads at this stage.
3. Microcentrifuge for 20 s, aspirate as much supernatant as possible.
4. Add 750 μL of 70% ethanol to the DNA pellet, centrifuge for 30 s, aspirate ethanol, air-dry the DNA (but do not let the DNA get too dry).

5. Add 500 μL TE buffer, gently dissolve the DNA for 3 h at 60°C, or 37°C overnight, and vortex it after incubation.
6. The quality of this DNA is good enough for PCR analysis in most cases. However, for the purpose of restriction enzyme digestion, or if more pure DNA is needed for another reason, after **step 2** the precipitated DNA should be fished out with a tip and transferred into a new Eppendorf tube with 750 μL of 70% ethanol. Then follow the same procedures described in **steps 4** and **5**.
7. The mouse tail DNA is analyzed with PCR. Three PCR primers are applied, a *neo*-primer, a wild-type primer, and a shared primer. The mutated allele will be amplified by the *neo*-primer and the shared primer, and the wild-type allele will be amplified by the wild-type primer and the shared primer. To increase the PCR efficiency, the size of the PCR products should be less than 1 kb, usually around 500 bp. The mutated allele and the wild-type allele should be distinguished easily.

3.4.3. Creating Targeted Mice with Uniform Genetic Background

The mutated allele should be bred onto a genetic background which allows its biological characterization. For many studies, a random or undefined genetic background may be appropriate for initial characterization as long as it is well controlled with litter mates. However, for some studies, an inbred background may be necessary. This can be done by iterative backcrossing with a pure wild-type strain. Six backcrosses produces animals that are ~96% congenic with the inbred backcross strain. It may be desirable to backcross further depending on the specific experiment.

3.4.4. Creating Mice with Multiple Targeted Genes

It may be desirable to test the phenotype of mice lacking multiple different chemokine receptors. In the case of chemokine receptors whose genes reside on separate chromosomes this can be accomplished by breeding single knockouts. In the case of chemokine receptors whose genes reside on the same chromosome, this is not feasible and direct methods of targeting multiple genes on the same construct or sequentially deriving multiple targeted alleles in ES cells must be employed. This is particularly relevant to the CC chemokine receptors.

3.4.5. Conditional Mutations

It is now possible to perform tissue-specific gene targeting ***(17)*** and inducible gene targeting ***(28)*** by using a Cre-lox recombination system. Cre is a recombinase from bacteriophage P1, which catalyzes DNA recombination between two sequence-specific 34-bp repeats called loxP ***(29,30)***. If a gene in a chromosome is engineered to be flanked by loxP, this gene can be deleted by the Cre recombinase.

Tissue-specific gene targeted mice can be generated in two steps. First, conventional gene targeting is used to generate mice in which the gene of interest is flanked by loxP sites. Then, the loxP-engineered mice are bred with animals

carrying a Cre transgene whose expression is under the control of a tissue-specific promoter. As a result, the offspring have a tissue-specific gene deletion.

With the same strategy, inducible gene targeted mice could be generated by breeding loxP-engineered mice to transgenic mice with a Cre gene whose expression can be induced by agents such as interferon α/β ***(28)*** or tetracycline ***(31)***. The gene can then be deleted when the offspring are exposed to the induction agent. Thus, conditional gene targeting can provide a powerful means to study the biological function of chemokine receptors.

References

1. Baggiolini, M., Dewald, B., and Moser, B. (1994) Interleukin-8 and related chemotactic cytokines—CXC and CC chemokines. *Adv. Immunol.* **55,** 97–179.
2. Baggiolini, M., Dewald, B., and Moser, B. (1997) Human chemokines: an update. *Annu. Rev. Immunol.* **15,** 675–705.
3. Cacalano, G., Lee, J., Kikly, K., Ryan, A. M., Pitts-Meek, S., Hultgren, B., et al. (1994) Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog. *Science* **265,** 682–684.
4. Cook, D. N., Beck, M. A., Coffman, T. M., Kirby, S. L., Sheridan, J. F., Pragnell, I. B., and Smithies, O. (1995) Requirement of MIP-1 alpha for an inflammatory response to viral infection. *Science* **269,** 1583–1585.
5. Gao, J. L., Wynn, T. A., Chang, Y., Lee, E. J., Broxmeyer, H. E., Cooper, S., et al. (1997) Impaired host defense, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. *J. Exp. Med.* **185,** 1959–1968.
6. Gerard, C., Frossard, J. L., Bhatia, M., Saluja, A., Gerard, N. P., Lu, B., Steer, M. (1997) Targeted disruption of the beta-chemokine receptor CCR1 protects against pancreatitis-associated lung injury. *J. Clin. Invest.* **100,** 2022–2027.
7. Forster, R., Mattis, A. E., Kremmer, E., Wolf, E., Brem, G., and Lipp, M. (1996) A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* **87,** 1037–1047.
8. Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., et al. (1996) Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* **382,** 635–638.
9. Kuziel, W. A., Morgan, S. J., Dawson, T. C., Griffin, S., Smithies, O., Ley, K., and Maeda, N. (1997) Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proc. Natl. Acad. Sci. USA* **94,** 12,053–12,058.
10. Kurihara, T., Warr, G., Loy, J., and Bravo, R. (1997) Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. *J. Exp. Med.* **186,** 1757–1762.
11. Boring, L., Gosling, J., Chensue, S. W., Kunkel, S. L., Farese, R. V., Jr., Broxmeyer, H. E., and Charo, I. F. (1997) Impaired monocyte migration and

reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J. Clin. Invest.* **100,** 2552–2561.
12. Lu, B., Rutledge, B. J., Gu, L., Fiorillo, J., Lukacs, N. W., Kunkel, S. L., et al. (1998) Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J. Exp. Med.* **187,** 601–608.
13. Smithies, O., Gregg, R. G., Boggs, S. S., Koralewski, M. A., and Kucherlapati, R. S. (1985) Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature* **317,** 230–234.
14. Thompson, S., Clarke, A. R., Pow, A. M., Hooper, M. L., and Melton, D. W. (1989) Germ line transmission and expression of a corrected HPRT gene produced by gene targeting in embryonic stem cells. *Cell* **56,** 313–321.
15. Evans, M. J. and Kaufman, M. H. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292,** 154–156.
16. Thomas, K. R. and Capecchi, M. R. (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51,** 503–512.
17. Gu, H., Marth, J. D., Orban, P. C., Mossmann, H., and Rajewsky, K. (1994) Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* **265,** 103–106.
18. Gu, H. (1994) Gene targeting and its application to the study of B-cell development. *Curr. Opin. Immunol.* **6,** 308–312.
19. Jung, S., Rajewsky, K., and Radbruch, A. (1993) Shutdown of class switch recombination by deletion of a switch region control element. *Science* **259,** 984–987.
20. Hasty, P., Ramirez-Solis, R., Krumlauf, R., Bradley, A. (1991) Introduction of a subtle mutation into the Hox-2.6 locus in embryonic stem cells. *Nature* **350,** 243–246.
21. Valancius, V. and Smithies, O. (1991) Testing of "in-out" targeting procedure for making subtle genomic modifications in mouse embryonic stem cells. *Mol. Cell. Biol.* **11,** 1402–1408.
22. Hasty, P. and Bradley, A. (1993) Gene targeting vectors for mammalian cells, in *Gene Targeting: A Practical Approach* (Joyner, A. L., ed.), Oxford University Press, New York, NY, pp. 1–31.
23. Gao, J. L. and Murphy, P. M. (1995) Cloning and differential tissue-specific expression of three mouse beta chemokine receptor-like genes, including the gene for a functional macrophage inflammatory protein-1 alpha receptor. *J. Biol. Chem.* **270,** 17,494–19,501.
24. Kozak, C. A., Gao, J. L., and Murphy, P. M. (1995) Mapping of the mouse macrophage inflammatory protein-1 alpha receptor gene Scya3r and two related mouse beta chemokine receptor-like genes to chromosome 9. *Genomics* **29,** 294–296.
25. Tybulewicz, V. L., Crawford, C. E., Jackson, P. K., Bronson, R. T., and Mulligan, R. C. (1991) Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. *Cell* **65,** 1153–1163.
26. Papaioannou, V. and Johnson, R. (1993) Production of chimeras and genetically defined offspring from targeted ES cells, in *Gene Targeting: A Practical Approach* (Joyner, A. L., ed.), Oxford University Press, New York, NY, pp. 107–144.

27. Wood, S. A., Allen, N. D., Rossant, J., Auerbach, A., and Nagy, A. (1993) Non-injection methods for the production of embryonic stem cell-embryo chimaeras. *Nature* **365,** 87–89.
28. Kuhn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995) Inducible gene targeting in mice. *Science* **269,** 1427–1429.
29. Sauer, B. and Henderson, N. (1988) Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc. Natl. Acad. Sci. USA* **85,** 5166–5170.
30. Sternberg, N. and Hamilton, D. (1981) Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. *J. Mol. Biol.* **150,** 467–486.
31. Furth, P. A., Stonge, L., Boger, H., Gruss, P., Gossen, M., Kistner, A., Bujard, H., and Hennighausen, L. (1994) Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. *Proc. Natl. Acad. Sci. USA* **91,** 9302–9306.

31

Measurement of Eosinophil Accumulation In Vivo

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1. Introduction

Eosinophils are leukocytes that contain characteristic cationic proteins in their granules that bind the acidic dye eosin. In contrast to neutrophils, eosinophils are minority cells in the blood and are predominantly tissue-dwelling cells found at sites in contact with the environment: the mucosal surfaces of the lung, gastrointestinal tract, and genitourinary tract. Selective accumulation of eosinophils, as opposed to neutrophils, is one of the major pathological features of the inflammatory response to infection with parasitic helminths, and in several diseases such as asthma, allergic rhinitis, and atopic dermatitis. A key step in leukocyte recruitment is the local production of chemoattractant molecules that orchestrate the adhesive interactions between leukocytes and the vascular endothelium.

To identify endogenous eosinophil chemoattractants, we developed an in vivo method to measure the accumulation of intravenously-injected radiolabeled eosinophils, at multiple skin sites injected with putative mediators ***(1)***. Using this bioassay to monitor the purification of eosinophil chemoattractant activity in bronchoalveolar lavage fluid from allergen-challenged guinea pigs, we discovered a novel chemokine, which we called "eotaxin" ***(2)***. The in vivo bioassay was based on our earlier work with the late Dr. Marc Rampart investigating the accumulation of ^{111}Indium-labeled neutrophils ***(3)***. To date, we have used similar methods to measure the accumulation of ^{111}In-eosinophils in guinea pig ***(1)***, rat ***(4)*** and, mouse ***(5)***, and ^{111}In-neutrophils in rabbit ***(3)*** and guinea pig ***(6)***. We have also used a nonradioactive method to measure the enzymic activity of eosinophil peroxidase (EPO) in extracts of skin and lung as an index of eosinophil accumulation ***(7,8)***.

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In this chapter, we describe the radioactive and enzymic methods used to measure the accumulation of eosinophils in guinea pig skin. The ^{111}In-labeled cell method provides results on the same day as the in vivo assay. This is especially useful when monitoring activity in HPLC fractions during purification of eosinophil chemoattractants because the speed of the assay facilitates progression to the next stage of purification. Although the EPO method takes at least one extra day to obtain results, it has the advantage that the eosinophils measured have not been subjected to isolation procedures which can modify their response to chemoattractant mediators. We first describe the isolation and purification of the eosinophils required for both methods: as a source of cells to radiolabel and to provide a standard curve for the EPO assay. We then describe the ^{111}In-labeled cell and EPO methods.

2. Materials

2.1. Purification of Guinea Pig Eosinophils

1. Animals: Dunkin-Hartley guinea pigs are used in our studies. Female exbreeder animals, 800–900 g, can be used as cell donors.
2. Acid Citrate Dextrose (ACD): Dissolve 2.1 g di-sodium hydrogen citrate and 2.5 g D-glucose in 50 mL of sterile distilled water, filter (0.2 microns) and autoclave (at the relatively low temperature of 90°C, to prevent degradation of the glucose). Store at 4°C.
3. Horse serum (Life Technologies, Paisley, Renfrewshire, UK). Store in aliquots at –20°C.
4. Sterile, pyrogen-free isotonic saline solution (Steriflex; Boots Co., Nottingham, UK).
5. Pyrogen- and preservative-free heparin sodium (1000 IU/mL; Paines & Byrne Ltd., Pabyrn Laboratories, Greenford, Middlesex, UK).
6. Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden). Store at 4°C.
7. Kimura Stain. First make 4 stock solutions:
   a. Toluidine blue solution: dissolve 0.05 g of Toluidine blue (Sigma), in 50 mL of 1.8% NaCl solution and 11 mL 96% ethanol. Add distilled water to a final vol of 100 mL;
   b. Light green solution: 0.03% Light green yellowfish (Sigma) in distilled water;
   c. Saponin, saturated solution: keep adding saponin (Sigma) to 50% ethanol until the suspension becomes orange;
   d. Phosphate buffer, pH 6.4: Mix 24.6 mL 67 m*M* Na_2HPO_4 and 75.4 mL 67 m*M* KH_2PO_4.

   To prepare the Kimura stain: Mix 5.5 mL of Toluidine blue solution, 0.4 mL of light green solution, 0.25 mL of saponin solution and 2.5 mL 67 m*M* phosphate buffer. Filter and keep at room temperature, protected from the light (covered with aluminium foil). Prepare weekly.
8. Prepare daily: HBSS/BSA: 10 mL sterile 10X Hank's balanced salt solution (HBSS, with phenol red, without Ca^{2+}/Mg^{2+}; Life Technologies, Bethesda, MD),

3 mL sterile 1 *M* HEPES (Life Technologies) and 0.25 g BSA (low in bacterial endotoxin; Sigma) made up to 100 mL with sterile distilled water. Adjust the pH to 7.3 (*see* **Note 1**).

2.2. Labeling and Accumulation of ^{111}In-eosinophils

1. Animals: Dunkin-Hartley guinea pigs (300–400 g).
2. $^{111}InCl_3$ (2 mCi in 0.2 mL sterile, pyrogen-free 0.04 N HCl; Amersham International, Amersham, Bucks, UK). Store in a thick lead container (*see* **Note 2**).
3. 2-Mercaptopyridine-N-oxide (Sigma): Dissolve 0.4 mg/mL in 50 m*M* PBS, pH 7.4, and keep dark at 4°C for up to 1 mo.
4. Citrated plasma: Kill the guinea pigs by CO_2-induced asphyxia. (To reduce the number of animals used, citrated plasma for future experiments may be prepared from the eosinophil donors, as in **Subheading 3.1.2.**) Take blood into ACD by cardiac puncture (2 mL ACD per 8 mL blood). Mix and centrifuge (2800*g*, 10 min, 20°C). Collect the supernatant and discard the pellet. Repeat the centrifugation and store the cell-free citrated plasma in aliquots at –20°C.
5. Hypnorm (0.315 mg/mL fentanyl citrate and 10 mg/mL fluanisone; Janssen Pharmaceutical Ltd., Grove, Oxford, UK).
6. Sagatal (60 mg/mL pentobarbitone sodium; May and Baker, Dagenham, Essex, UK).
7. Prepare daily: HBSS/citrated plasma: 9 mL 10X HBSS w/o Ca^{2+}/Mg^{2+}, 3 mL 1 *M* HEPES and 10 mL of cell-free citrated guinea pig plasma made up to 100 mL with sterile distilled water. Adjust the pH to 7.3 (*see* **Note 1**).

2.3. Measurement of EPO in Skin

1. 0.5% Hexadecyltrimethylammonium bromide (HTAB; Sigma) in PBS (137 m*M* NaCl, 2.68 m*M* KCl, 7.98 m*M* Na_2HPO_4, 1.47 m*M* KH_2PO_4, pH 7.2; Life Technologies).
2. 1.6% and 0.2% NaCl.
3. 0.1*M* Tris-HCl buffer pH 8.0. Store at 4°C.
4. 3% H_2O_2 (Sigma). Store in aliquots at 4°C.
5. 4 *M* sulphuric acid.
6. Prepare daily: EPO substrate solution: Dissolve a 20 mg tablet of o-phenylenediamine dihydrochloride (OPD, Sigma) in 12.8 mL Tris/HCl buffer (*see* **Note 3**). Prepare fresh and keep dark. Just before addition to the samples, add 43 µL of 3% H_2O_2.

3. Methods

3.1. Purification of Guinea Pig Eosinophils

Due to the low number of eosinophils in peripheral blood, especially in guinea pigs, eosinophils are elicited in the peritoneal cavity by repeated injection of horse serum. Eosinophils are then purified to >95% by use of a discontinous Percoll gradient. The yield of eosinophils varies but we usually use 1 exbreeder donor for every 2–4 recipients.

1. Inject 1 mL warm (30–37°C) horse serum into the peritoneal cavity of the donor guinea pigs. Repeat at 2-d intervals for a total of 5 injections. If a steady supply of eosinophil donors is required, peritoneal eosinophilia can now be maintained by weekly injections of horse serum (best results after a total of 7 injections). Humanely kill the animals by CO_2-induced asphyxia 36–48 h (minimum 24 h) after the last injection to decrease neutrophil contamination.
2. If you wish, blood may be taken at this stage for the preparation of citrated plasma for future use (see **Subheading 2.2.4.**).
3. Remove a small strip of skin from the abdomen and make a small incision in the peritoneal wall. Inject 35 mL of heparinized saline (10 IU/mL) and gently massage the abdomen for 20 s. Extend the incision and harvest the cell suspension.
4. Centrifuge (250*g*, 7 min, 20°C) to collect the cells.
5. Prepare the discontinuous Percoll gradients while the cells are spinning down (*see* **Note 4**). Take care to avoid turbulence by adding each Percoll density layer to the side of the tube and not directly to the preceding layer. The details given are sufficient for one gradient tube which, in turn, is sufficient for the cells from one donor. Mix 9 mL of the Percoll stock and 1 mL sterile 10X HBSS w/o Ca^{2+}/Mg^{2+} and adjust to pH 7.3 (*see* **Note 1**). Take 5 tubes and prepare the different densities for the gradient (*see* **Table 1**). Add 2 mL of Percoll density 1.100 to a suitable centrifuge tube. Attach a 19-gauge needle to a 2 mL syringe and fix it so that the tip of the needle is touching the inner wall at the top of the gradient tube. Carefully add the second layer (3 mL at density 1.095). Continue with the procedure (**Table 1**) until all the layers are in place.
6. Gently pour off the supernatant from the centrifuged cells and resuspend the pellet in 3 mL HBSS/BSA. Using a sterile plastic pastette, carefully layer the cell suspension onto the Percoll gradient by adding to the side wall of the tube.
7. Centrifuge at 1500*g*, 25 min, 20°C without the brake.
8. Once centrifuged, the eosinophils are found at the two lower interfaces (1.090/1.095 and 1.095/1.100). Using a plastic pastette, carefully pipet off the less-dense layers (containing mononuclear cells). Using a new pastette, remove the eosinophil layers and wash by the addition of 10 mL HBSS/BSA, gentle mixing, and centrifugation (250*g*, 7 min, 20°C).
9. Discard the supernatant and resuspend the cell pellet in 1 mL of HBSS/BSA. Mix 10 μL of the cell suspension with 90 μL of Kimura stain. With this stain, eosinophils have a green cytoplasm while mononuclear cells and neutrophils do not (all cells have a blue nucleus). Count the different cell types in a haemocytometer. Eosinophil purity should be >95%: the predominant contaminating cells are mononuclear and the major exclusion criterion for the majority of our studies is the presence of >1% neutrophils.

3.2. Labeling and Accumulation of ^{111}In-eosinophils

The ^{111}Indium/2-mercaptopyridine-N-oxide chelate penetrates the cell and remains cell-bound. Nonbound ^{111}Indium is removed with three successive washes. During the preparation of labeled cells (*see* **Subheading 3.2.1.**),

Table 1
Preparation of the Discontinuous Percoll Gradient

Layer	Density (g/mL)	Percoll/HBSS solution (mL)	HBSS/BSA (mL)	Volume per gradient (mL)
1	1.100	1.60	0.41	2
2	1.095	3.00	0.94	3
3	1.090	2.74	1.26	3
4	1.085	1.25	0.75	2
5	1.080	1.14	0.86	2

prepare the recipient guinea pigs and samples for intradermal injection (*see* **Subheading 3.2.2.**). *Use gloves, safety glasses, and lead shielding while handling radioactive material.*

3.2.1. Labeling

1. To 100 µL of 2-mercaptopyridine-N-oxide, shielded by lead, add 10 µL of $^{111}InCl_3$ (approx 2–4 MBq). The vol of $^{111}InCl_3$ can be increased to 20 µL if the label is 3 or more days past its activity date (*see* **Note 2**). Incubate in the dark for 10 min at room temperature.
2. Add the 1 mL cell suspension (maximum 2×10^7 cells, which is sufficient for 4 recipient guinea pigs) to the ^{111}In-chelate. Mix gently and incubate for 15 min at room temperature.
3. Meanwhile, prepare the HBSS/citrated plasma (*see* **Subheading 2.2.4.**) and five tubes (*see* **Note 5**) labeled: T, S_1, S_2, S_3 (each filled with 990 µL HBSS/BSA) and T_{10} (filled with 900 µL HBSS/BSA). These tubes will be used to assess the labeling efficiency (S_1–S_3) and to convert radioactive counts/minute into number of ^{111}In-labeled eosinophils (T_{10}).
4. After the 15-min cell incubation, add 9 mL of HBSS/citrated plasma, mix and centrifuge (250*g*, 7 min, 20°C). Add 10 µL of the supernatant to tube S_1 and, using a plastic pastette, remove the remaining supernatant into radioactive waste. Repeat this wash step twice: in each case resuspend the labeled cells in 1 mL HBSS/citrated plasma, transfer to a new tube, add a further 9 mL of HBSS/citrated plasma and take 10 µL of the supernatant (to give S_2 and S_3).
5. Resuspend the washed cell pellet in 1 mL of HBSS/citrated plasma. Remove 10 µL of the cell suspension into tube T. Mix and dilute further by adding 100 µL to tube T_{10}.
6. Using the cell counts from **Subheading 3.1.9.**, adjust the eosinophil concentration to 10^7/mL with HBSS/citrated plasma.

3.2.2. Intravenous Injection and Accumulation in Skin

1. Sedate recipient guinea pigs (300–400 g) with hypnorm (0.2 mL, intramuscular).
2. Shave the dorsal skin and mark the sites for intradermal injections in duplicate according to a balanced site injection plan.

3. Prepare the agents for intradermal injection, e.g., chemokines or HPLC fractions, in HBSS/BSA.
4. If the animals need a systemic pretreatment, inject the substance before injecting the radiolabeled cells (e.g., antibodies given intravenously 15 min before the labeled cells).
5. Inject 0.5 mL of the ^{111}In-eosinophil suspension (5×10^6 cells/guinea pig) via the marginal ear vein (the hind foot dorsal vein can also be used). We use 27-gauge needles.
6. Fill labeled sterile syringes with the test agents for the intradermal injections.
7. Five minutes after injecting the labeled cells, begin the intradermal injections (0.1 mL/site).
8. After a predetermined time period of eosinophil accumulation, anesthetize the animals with Sagatal (60 mg/kg). Collect a blood sample (at least 3 mL) into heparin (10 IU/mL) by cardiac puncture and humanely kill the animal with an overdose of Sagatal.

3.2.3. Counting

1. Remove the back skin, trim any loose subcutaneous tissue and snip the subdermal layer. Massage blood from the skin with damp tissue paper. Be sure that no blood remains in the small vessels as the ^{111}In-eosinophils in this blood will be counted as tissue ^{111}In-eosinophils.
2. Place the skin, with its external surface uppermost, on a square of parafilm. Punch out the skin sites with a 17-mm diameter punch. Place each skin site at the bottom of previously labeled tubes (*see* **Note 5**).
3. Spin two 1-mL aliquots of the blood sample in a microfuge. Take 500 µL plasma from the first, mix with 500 µL of HBSS/BSA ("tube 500," to determine free $^{111}In^{3+}$ in the plasma) and discard the cell pellet. Take duplicate 100 µL samples of plasma from the second blood aliquot and mix each with 900 µL HBSS/BSA ("tubes 100," *see* **Note 6**). Then remove the remaining plasma and use the blood cell pellet to calculate the number of circulating ^{111}In-eosinophils.
4. Put the different samples to count in a gamma-counter in the following order: T_{10}, S_1, S_2, S_3, blood cell pellet, tube 500, duplicate tubes 100, and duplicate tubes of skin sites 1, 2, and so on.

3.2.4. Calculations

CPM/^{111}In-eosinophil:
(CPM of T_{10}/Number of eosinophils labeled) $\times 10^3$

% Labeling Efficiency:
(CPM of T_{10}/(CPM of $T_{10} + S_1 + S_2 + S_3$)) $\times 10^2$

% Circulating Eosinophils:
((CPM in 1 mL blood cell pellet × body blood volume)/CPM injected) $\times 10^2$

Where body blood volume (mL) is calculated as 8% of body weight (g) and CPM injected is CPM of $T_{10} \times 5 \times 10^9$/Number of eosinophils labelled

% ^{111}In free in plasma:
(CPM of tube 500/CPM of (blood cell pellet + tube 500)) × 10^2

^{111}In-eosinophils/skin site:
CPM in skin site/CPM per ^{111}In-eosinophil

In general, the labeling efficiency is > 80%. Clearly, it is desirable to have a low level of ^{111}Indium free in the plasma, otherwise plasma extravasation will contribute significantly to the measurement of ^{111}In-eosinophil recruitment.

Before beginning studies of ^{111}In-eosinophil accumulation, it is recommended that the circulating ^{111}In-eosinophils and %^{111}In free in the plasma are measured as a function of time. Circulating ^{111}In-eosinophils will decrease because of clearance mechanisms and % free label will increase with time. We have found that a 2–4 h accumulation period gives satisfactory results.

3.3. Measurement of EPO in Skin

This method of measuring eosinophil accumulation in vivo has the advantage that it does not require the use of radioactive material. However, it is less sensitive to low doses of chemoattractant, although this can be overcome by the intravenous injection of IL-5 to boost the numbers of eosinophils in the circulation *(7)*. The isolation of eosinophils, for use as a standard, and the preparations for intradermal injections have been described in **Subheadings 3.1.** and **3.2.2.** and will not be repeated here.

3.3.1. Extraction of EPO from Skin

1. If required, inject IL-5 (1 μg/kg, 1 h before intradermal injections of test substances) to boost the circulating eosinophil numbers.
2. Inject eosinophil chemoattractants intradermally (*see* **Subheading 3.2.2.**).
3. After an appropriate eosinophil accumulation period, kill the animals by CO_2-induced asphyxia. Remove, clean, and excise the skin injection sites, as described in **Subheading 3.2.3.**, and freeze at –80°C.
4. During thawing, slice the skin sites into 10 strips and add to 4 mL 0.5% HTAB.
5. Homogenize thoroughly and sonicate for 10 s.
6. Perform 2 more cycles of freeze/thawing (samples can be stored frozen at any stage of this procedure). Finally, freeze the samples again at –80°C.

3.3.2. Preparation of EPO Standard

1. Prepare guinea pig eosinophils as described in **Subheading 3.1.** with the following additional steps.
2. To lyse any remaining red blood cells (which alter the conversion of substrate), resuspend the cells in at least 5 mL 0.2% NaCl for 20 s and add an equal vol of 1.6% NaCl. Centrifuge (250g, 7 min) and discard the supernatant.

3. Resuspend the cells in HBSS/BSA and count (**Subheading 3.1.9.**). The concentration should be at least 10^7 eosinophils/mL.
4. Add 0.5% HTAB to a final eosinophil concentration of 10^6/mL. Sonicate for 10 s, freeze/thaw twice, and then freeze in 1-mL aliquots.

3.3.3. Measurement of EPO Activity

1. Centrifuge skin extracts (2800*g*, 10 min) and then centrifuge an aliquot of the supernatant in a microfuge (13,000*g*, 20 min). Similarly, microfuge an aliquot of the standard.
2. Recover the supernatants and incubate at 60°C for 2h (to retain the EPO activity but denature other proteins which interfere with the enzyme assay). Repeat the microfuge step.
3. Construct the EPO calibration curve by 1/20 dilution in 0.5% HTAB (to give 5×10^4 eosinophils/mL) and thereafter serial twofold dilutions down to 780 eosinophils/mL.
4. Place the standards, skin extracts (diluted in 0.5% HTAB if necessary), and 0.5% HTAB as the blank in duplicate wells (100 µL/well) in a 96-well plate.
5. Add freshly prepared substrate solution (100 µL/well) and incubate dark for 30 min at room temperature (wrapped in aluminum foil).
6. Add 4 *M* H_2SO_4 (50-µL/well) to stop the reaction and enhance the absorbance at 492 nm, which is read on a microtiter plate reader.
7. Construct the standard curve and determine the eosinophil content of the skin extracts.

4. Notes

1. Do not put a pH probe into the HBSS mixtures to be used for eosinophil purification and labeling. Adjust the pH of HBSS/BSA and HBSS/citrated plasma by adding 0.1 *M* NaOH until the color of the phenol red indicator in the HBSS shows pH 7.3. (Remove an aliquot to check the pH and then discard.) Adjust the pH of Percoll/HBSS by cautious addition of 1 *M* HCl with mixing to prevent formation of a precipitate. Use the color of the phenol red indicator as a guide, but only remove a small aliquot for pH measurement if the pH probe will function with 100 µL or less (and then discard).
2. ^{111}Indium has a half-life of 2.8 d, which means that all the experiments need to be completed within 1 wk of the activity date.
3. It is important that the incubation of EPO extracts (in HTAB/PBS) with substrate are at pH 8.0. It is recommended to check the final pH of a representative reaction mixture, especially if higher concentrations of the OPD dihydrochloride are to be tested.
4. We routinely use a (dedicated) laminar flow cabinet to prepare the Percoll gradients and perform subsequent manipulations. It is important to avoid activating the cells during these procedures.
5. All samples to be counted in the gamma counter should have approximately similar geometry. In our automatic 10-head counter (Canberra Packard Ltd., Pangbourne, Berks, UK), we use 2 mL screw-capped Eppendorf tubes (filled to

1 mL) for liquid samples and cheaper tubes for skin sites (polycarbonate tubes with the skin pushed down to occupy the bottom portion).

6. We use tubes "100" for determination of plasma levels of intravenously injected ^{125}I-albumin in experiments in which plasma protein accumulation is measured in the same skin sites as ^{111}In-eosinophils (using cross channel correction for the spillover of one isotope into the energy window used to measure the other isotope). Therefore to comply with the tube order programmed into our gamma counter, tubes "100" (or blank positions, when measurement of plasma protein accumulation is not required) are always utilized.

References

1. Faccioli, L. H., Nourshargh, S., Moqbel, R., Williams, F. M., Sehmi, R., Kay, A. B., and Williams, T. J. (1991) The accumulation of ^{111}In-eosinophils induced by inflammatory mediators in vivo. *Immunology* **73,** 222–227.
2. Jose, P. J., Griffiths-Johnson, D. A., Collins, P. D., Walsh, D. T., Moqbel, R., Totty, N. F., et al. (1994) Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea-pig model of allergic airways inflammation. *J. Exp. Med.* **179,** 881–887.
3. Rampart, M. and Williams, T. J. (1988) Evidence that neutrophil accumulation induced by interleukin-1 requires both local protein biosynthesis and neutrophil CD18 antigen expression in vivo. *Br. J. Pharmacol.* **94,** 1143–1148.
4. Sanz, M.-J., Weg, V. B., Bolanowski, M. A., and Nourshargh, S. (1995) IL-1 is a potent inducer of eosinophil accumulation in rat skin: inhibition of response by a PAF antagonist and an anti-human IL-8 antibody. *J. Immunol.* **154,** 1364–1373.
5. Teixeira, M. M., Wells, T. N. C., Lukacs, N. W., Proudfoot, A. E. I., Kunkel, S. L., Williams, T. J., and Hellewell, P. G. (1997) Chemokine-induced eosinophil recruitment. Evidence of a role for endogenous eotaxin in an in vivo allergy model in mouse skin. *J. Clin. Invest.* **100,** 1657–1666.
6. Sanz, M.-J., Weg, V. B., Walsh, D. T., Williams, T. J., and Nourshargh, S. (1994) Differential effects of the PAF receptor antagonist UK-74,505 on neutrophil and eosinophil accumulation in guinea-pig skin. *Br. J. Pharmacol.* **113,** 513–521.
7. Collins, P. D., Marleau, S., Griffiths-Johnson, D. A., Jose, P. J., and Williams, T. J. (1995) Co-operation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. *J. Exp. Med.* **182,** 1169–1174.
8. Humbles, A. A., Conroy, D. M., Marleau, S., Rankin, S. M., Palframan, R. T., Proudfoot, A. E. I., et al. (1997) Kinetics of eotaxin generation and its relationship to eosinophil accumulation in allergic airways disease: analysis in a guinea pig model in vivo. *J. Exp. Med.* **186,** 601–612.

32

Murine Model of Allergic Lung Inflammation

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1. Introduction

Animal models of allergic lung inflammation have been developed in an attempt to better define the pathogenesis of asthma. Using these models, important mechanistic questions that are difficult to pursue through clinical investigation have been addressed. Classically guinea pigs were used, but during the last decade an increasing number of research groups have used murine models. This is due to the fact that many immunological tools to study immune mechanisms are available for mice, such as inbred mice strains, transgenic and gene deleted mice, antibodies against murine cellular markers, and recombinant murine soluble protein.

In general, BALB/c and C57BL/6 mice are sensitized with an antigen such as Chicken Egg Ovalbumin ***(1,2)***, aspergillus ***(3)***, house dust mite ***(4)***, or sheep red blood cells ***(5)***. This sensitization is often administered with an adjuvant such as alum ***(6)***. After sensitization mice are challenged, once, or repeatedly, with the antigen by inhalation or tracheal instillation. Several parameters can be measured in the treated mice such as antigen-specific IgE levels, airway hyperresponsiveness, cellular infiltration, protein levels in the bronchial lavage, and histological changes. Although animal models have limitations in terms of their application to humans, information obtained from the murine model has provided an insight into important immuno-pathological mechanisms. Much knowledge on the role of cytokines ***(5)***, chemokines ***(7)***, T cells ***(8)***, costimulatory molecules ***(9)***, and adhesion molecules ***(10)*** has been gained.

2. Materials

2.1. Mice

BALB/c, C57BL/6 mice or 129SV strain (*see* **Note 1**).

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2.2. Immunization and Challenge

2.2.1. Immunization: Without Adjuvant

1. Chicken Egg Ovalbumin, OVA (Sigma, no. A-5503), 10 g in 0.1 mL NaCl (0.9 % w/v). (B/Braun Medical A.G.; *see* **Note 2**).
2. 1-mL syringe.
3. $25G^{5/8}$ 0.5 × 16-mm needle.

2.2.2. Immunization: With Adjuvant

1. 50 μg of chicken egg ovalbumin (OVA) in 0.2 mL aluminium hydroxide (alum). For 1 mL mix: 25 μL OVA (2 mg/mL), 250 μL alum 1.3% in water (Serva, Heidelberg, Germany, no.12261), 725 μL NaCl (0.9%).
2. 1-mL syringe.
3. $25G^{5/8}$ 0.5 × 16-mm needle.

2.2.3. Challenge

1. Intranasal instillation: 50 μL OVA (2 mg/mL) in NaCl. Halothane (2-Brom-2-chlor-1, 1,1-trifluoroethane: Fluka, No. 16730).
2. Intratracheal instillation: 20 μg OVA (2 mg/mL) in 10 μL NaCl. Anesthetic solution (*see* **Note 3**).

2.3. Airway Hyperresponsiveness

1. Whole body plethysmography (Buxco®, EMKA Technologies, Paris). (**Fig. 1**).
2. Methacholine (Acetyl-β-methylcholine chloride 98%, Aldrich, Milwaukee, MI, no A1,800-1). For a dose response, four different dilutions are prepared: 6×10^{-2} *M*, 3×10^{-2} *M*, 1.5×10^{-2} *M*, and 0.75×10^{-2} *M* from a 1 *M* stock solution in NaCl stored at –20°C.

2.4. Bronchoalveolar Lavages and Differential Cell Counts

2.4.1. Bronchoalveolar Lavages

1. Urethane (Sigma, no. U-2500) 14% in NaCl 0.9%.
2. Polyethylene tubing, 0.76-mm internal diameter (Becton Dickinson, no.427416).
3. 1-mL syringe.
4. $25G^{5/8}$ 0.5 × 16 needle.
5. 1X PBS containing 10 m*M* EDTA, pH 8.0.
6. 3 mL Propylene tubes (Falcon, Los Angeles, CA).
7. Surgery material.

2.4.2. Differential Cell Counts

1. Cytospin centrifuge (Shandon, Pittsburgh, PA).
2. Superfrost microscope slides (Merck, Darmstadt, Germany).
3. Filter cards (Shandon).

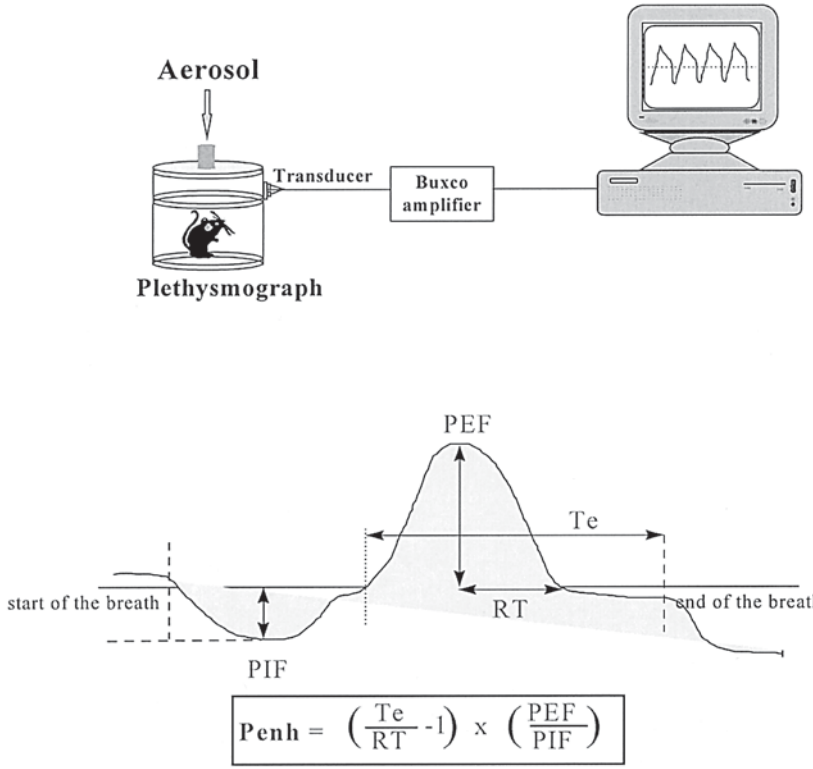

Fig. 1. Buxco® whole body plethysmograph for measuring lung function. The mouse is placed in a plexiglas chamber, where it is free to move. A port at the top of the chamber is connected to a nebulizer to allow administration of aerosolized chemicals. A transducer linked to an amplifier monitors the pressure changes within the chamber, which correspond to the animal's breathing rates and depths. These raw data are converted to a value called Penh, which is a measure of BHR. Readout consists of change in Penh over time. An increase in Penh indicates that the mouse is hyperresponsive.

4. Diff-Quik™ staining solutions (*see* **Note 4**).
5. Microscope.
6. Immersion oil (Fluka, no. 56822).

2.5. Lung Histology

2.5.1. Lung Sections

1. O.C.T. compound (Bayer, cat. no. 4583): embedding medium for frozen tissue specimens (*see* **Note 5**).

2. Braided, coated silk suture (B/Braun, no. 0113404/3).
3. Tissue-Tek cryomold, (Miles Laboratory, Rexdale, Ont., Canada).
4. Dry ice.
5. Superfrost microscope slides.
6. Cryostat.

2.5.2. Lung Histology

1. Methanol.
2. May-Grunwald solution (Fluka, no. 63590).
3. Giemsa solution (Fluka Chemika, no. 48900).
4. Phosphate-buffered saline (PBS).
5. Tris-HCl buffer (pH 7.6).
6. Diamino benzidine (DAB) substrate solution (*see* **Note 6**).
7. Periodic Acid Schiff's Base (PAS) (Sigma, no. 395-1 and 395-2).
8. Alcian blue (8GX-Sigma, no. A 3157).
9. Eukitt (Polylabo, Geneva, Switzerland, no. 82601).

2.5.3. Lung Immuno-Histochemistry

1. Rat antimouse MAbs (*see* **Note 7**).
2. Biotinylated-mouse $F(ab')_2$ anti-rat IgG antibodies (Southern Biotechnology Associates, Inc.)
3. Alkaline Phosphatase ABC kits (Vector Laboratories, Inc, Burlingame, CA).
4. Fast-Red substrate system (Dako).
5. Analytical grade glycerol.
6. Mowiol 4-88 (Hoechst).
7. Tris-HCl, pH 8.5.
8. DABCO (1,4 diazabicyclo-[2.2.2]-octane) (Fluka, no. 33480).

3. Methods

3.1. Immunization and Challenge

3.1.1. Without Adjuvant

1. Mice are immunized with 10 μg of ovalbumin (OVA) in 0.1 mL NaCl 0.9% administered intraperitoneally (ip) every other day for 14 d.
2. Forty days later, mice are anesthetized by inhaled halothane and instilled with 50 μg of ovalbumin (in 50 μL) intranasally for 5 d.
3. Alternatively, 20 μg of ovalbumin (in 10 μL) is delivered intratracheally ***(11)*** 3 times, at 3 d intervals.

3.1.2. With Adjuvant

1. Mice are immunized with 50 μg of OVA in 0.2 mL alum administered intraperitoneally (ip) on day 0.

2. Fourteen days later, mice are anesthetized by inhaled halothane and instilled with 50 μg of ovalbumin (in 50 μL) intranasally for 5 d.

3.2. Airway Hyperresponsiveness

Airway responsiveness is measured by recording respiratory pressure curves by whole body plethysmography in response to inhaled methacholine, for 20 s, using the XA 157 program. The data are analyzed using Excel or datanalyst software (*see* **Fig. 1**) ***(12)***.

3.3. Bronchoalveolar Lavages and Differential Cell Counts

3.3.1. Bronchoalveolar Lavages

1. Two days after the last challenge, the mice are anesthetized by ip injection of urethane (14 % in NaCl).
2. The lungs and trachea are revealed and an incision is made in the trachea.
3. A microsyringe fitted with a cannula fashioned from a blunted, hypodermic needle is gently inserted into the trachea. Four washes with 0.4 mL PBS/EDTA are made. The four washes are pooled and represent the bronchoalveolar lavages or BAL (*see* **Note 8**).

3.3.2. Differential Cell Counts

3.3.2.1. Total Cell Count

Twenty μL of the BAL is mixed with 20 μL of trypan blue, which stains the dead cells blue. Living cells are counted under a microscope using a Neubauer hemacytometer.

3.3.2.2. Cytospin and Staining

1. 100 μL of BAL are placed on a microscope slide and spun at 3000 rpm for 10 min in a Cytospin centrifuge.
2. Allow the slides to dry at room temperature for 1 h prior to staining.
3. Dip the slides in fixative solution 10 times.
4. Dip the slides in Diff-Quick® I solution, 10 times.
5. Dip the slides in Diff-Quick II® solution, 8 times.
6. Dry the slides at room temperature.
7. Count the cells (macrophages, eosinophils, lymphocytes, and neutrophils) under the microscope. Count 3 fields and calculate the mean.

3.4. Lung Histology

3.4.1. Frozen Lung Sections

1. After the broncholavage, the lungs are gently inflated by instillation of O.C.T. (700 μL) (*see* **Note 5**).

2. The cannula is then removed from the trachea, which is closed with silk suture thread.
3. The lung is immerged in a Tissue-Tek capsule containing O.C.T. and transferred to dry ice. Once the O.C.T. starts to freeze, cover the lung with O.C.T. (*see* **Note 9**).
4. Store the frozen lung at –80°C.
5. Lung sections of 10 μm are made using a cryostat and placed on Superfrost microscope slides.
6. Fix sections with methanol for 5 min.
7. Dry sections at room temperature.
8. Store slides at –20°C (*see* **Note 10**).

3.4.2. Lung Histology

3.4.2.1. May-Grunwald-Giemsa (*see* **Note 11**)

Slides are:

1. Incubated for 2.5 min in methanol;
2. Incubated for 5 min in 50% May-Grunwald solution (*see* **Note 12**);
3. Incubated for 10 min in 7% Giemsa solution;
4. Rinsed with running tap water;
5. Dried completely in air;
6. Mounted with Eukitt (*see* **Note 13**).

3.4.2.2. DAB Staining for Eosinophils (*see* **Note 14**)

1. Surround the sections with a pan pen (this prevents wasting valuable reagents by keeping liquid pooled in a small droplet).
2. Rehydrate the sections in 50 m*M* Tris buffer pH 7.5 for 15 min.
3. Add 200 μL of DAB (Sigma, no. D-5637) on to the section and leave 15 min.
4. Wash 5 times with Tris buffer.
5. Counter stain with Methyl Green or toluidine blue.
6. Mount with Eukitt.

3.4.2.3. Alcian Blue/Periodic Acid Schiff's Base Stain for Mucus

1. Fix slides in 2% paraformaldehyde/PBS for 30 min at room temperature.
2. Rinse slides three times with PBS.
3. Stain slides with 1% Alcian blue in 3% acetic acid pH 2.5, for 10 min at room temperature.
4. Rinse slides for 10 min with running tap water.
5. Incubate slides for 5 min at room temperature in periodic acid (1% aqueous solution).
6. Rinse slides in several changes of deionized water.
7. Incubate slides in Schiff's reagent for 15 min at room temperature.
8. Rinse slides in running tap water for 5 min.
9. Mount with Eukitt.

3.4.3. Immunohistochemistry

1. Hydrate sections in PBS.
2. Incubate sections for 30 min at 20°C with fluorescein-conjugated primary rat antibody directed against mouse antigens. If nonconjugated antibodies are used in the first incubation, follow by fluorescein-conjugated mouse $F(ab')_2$ anti-rat IgG antibodies.
3. Mount with Mowiol solution:
   a. Place 6 g of analytical grade glycerol in a disposable plastic conical tube.
   b. Add 2.4 g Mowiol to the glycerol and stir.
   c. Add 6 mL distilled water to the solution and leave for 3 h at room temperature.
   d. Add 12 mL of 0.2 *M* Tris buffer, pH 8.5, and incubate the solution at 50°C for 1–2 h with occasional stirring to dissolve the Mowiol (*see* **Note 15a**).
   e. Aliquot the mounting medium into small fractions and store in airtight containers at –20°C (*see* **Note 15b**).
   f. Add 2.5% DABCO to obtain the working solution (*see* **Note 15c**).

3.5. Treatment

Antibodies against chemokines or chemokine antagonists are injected 30 min before each intranasal Ova-challenge, either ip, in a vol of 100 µL, or intranasally in a vol of 50 µL, or intratracheally in a volume of 10 µL

4. Notes

1. Eight-week-old female mice.
2. 2 mg/mL stock solution in 0.9% NaCl is prepared, aliquoted (0.5 mL aliquots) and stored at –20°C.
3. Anesthetic solution: For 10 mL: 200 µL Dormicum (Roche) 5 mg/mL; 625 µL Rompun (Bayer) 2% solution; 5 mL Ketasol-50 (Graeub) 50 mg/mL; PBS: add to a final vol of 10 mL; Inject 50 µL per 10 g of body weight.
4. Diff-Quick (Baxter Diagnostics) consists of three different solutions:
   a. Diff-Quick Fix: fixative solution (Fast green in methanol, 0.002 g/L).
   b. Diff-Quick I: eosin G in phosphate buffer, pH 6.6.
   c. Diff-Quick II: thiazine in phosphate buffer, pH 6.6.
5. Use TissueTek® O.C.T. compound (Miles Inc.) to bind tissue to the specimen block and to surround and cover the tissue specimen.
6. DAB substrate solution is 0.5 mg/mL DAB, 1% hydrogen peroxide in 50 m*M* Tris-HCl, pH 7.6.
7. Rat MAb against murine antigen.
8. After the Cytospin centrifugation and the total cell count, the remaining BAL is centrifuged for 10 min at 425*g*. The volume of the supernatant is measured and frozen at –20°C. The BAL fluid can be used to test for the presence of different molecules such as cytokines by ELISA. The cell pellet can also be kept for RNA analysis.

9. The lung should not be frozen without O.C.T. The O.C.T protects the tissue and it is important to have intact tissue for the histological analysis.
10. Sections can be stored at –20°C for several months.
11. May-Grunwald-Giemsa staining will show all of the infiltrating cells in the lung.
12. May-Grunwald solution should be diluted with an equal vol of distilled water and the Giemsa solution should be diluted to 7% in distilled water before use.
13. Put one drop of Eukitt on the section and cover with a cover slip.
14. To prepare the DAB solution:
    a. Add 25 mg DAB to 50 mL Tris buffer, 50 m*M*, pH 7.5.
    b. Filter.
    c. Add 50 μL H_2O_2, 30%, just before use.
15. Mowiol:
    a. If the mixture needs further clarification, it can be centrifuged at 5000*g* for 15 min.
    b. The preparation seems to remain stable for several months, but if it becomes cloudy during storage a fresh batch should be prepared.
    c. The working aliquot will remain stable at room temperature for several weeks but again, use a fresh aliquot if the solution becomes cloudy.

References

1. Blyth, D. I., Pedrick, M. S., Savage, T. J., Hessel, E.M., and Fattah, D. (1996) Lung inflammation and epithelial changes in a murine model of atopic asthma. *Am. J. Respir. Cell Mol. Biol.* **14,** 425–438.
2. Chvatchko, Y., Kosco-Vilbois, M. H., Herren, S., Lefort, J., and Bonnefoy, J.-Y. (1996) Germinal center formation and local immunoglobulin E (IgE) production in the lung after an airway antigenic challenge. *J. Exp. Med.* **184,** 2353–2360.
3. Kurup, V. P., Grunig, G., Knutsen, A. P., and Murali, P. S. (1998) Cytokines in allergic bronchopulmonary aspergillosis. [Review] *Res. Immunol.* **149,** 466–477.
4. O'Brian, R., Ooi, M. A., Clarke, A. H., and Thomas, W. R. (1996) Immunologic responses following respiratory sensitization to house dust mite allergens in mice. [Review] *Immunol. Cell Biol.* **74,** 174–179.
5. Coyle, A. J., Le Gros, G., Bertrand, C., Tsuyuki, S., Heusser, C. H., Kopf, M., and Anderson, G. P. (1995) Interleukin-4 is required for the induction of lung Th2 mucosal immunity. *Am. J. Respir. Cell Mol. Biol.* **13,** 54–59.
6. Coyle, A. J., Wagner, K., Bertrand, C., Tsuyuki, S., Bews, J., and Heusser, C. (1996) Central role of immunoglobulin (Ig) E in the induction of lung eosinophil infiltration and T helper 2 cell cytokine production: inhibition by a non-anaphylactogenic anti-IgE antibody. *J. Exp. Med.* **183,** 1303–1310.
7. Gonzalo, J. A., Lloyd, C. M., Wen, D., Albar, J. P., Wells, T. N., Proudfoot, A., et al. (1998) The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J. Exp. Med.* **188,** 157–167.
8. Li, X. M., Schofield, B. H., Wang, Q. F., Kim, K. H., and Huang, S. K. (1998) Induction of pulmonary allergic responses by antigen-specific Th2 cells. *J. Immunol.* **160,** 1378–1384.

9. Tsuyuki, S., Tsuyuki, J., Einsle, K., Kopf, M., and Coyle, A. J. (1997) Costimulation through B7-2 (CD86) Is required for the induction of a lung mucosal T helper cell 2 (Th2) immune response and altered airway responsiveness. *J. Exp. Med.* **185,** 1671–1679.
10. Gonzalo, J. A., Lloyd, C. M., Kremer, L., Finger, E., Martinez, A., Siegelman, M. H., et al. (1996) Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *J. Clin. Invest.* **98,** 2332–2345.
11. Ho, W. and Furst, A. (1973) Intratracheal instillation method for mouse lungs. *Oncology* **27,** 385–393.
12. Hamelmann, E., Schwarze, J., Takeda, K., Oshiba, A., Larsen, G. L., Irvin, C. G., and Gelfand, E. W. (1997) Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* **156,** 766–775.

33

Murine Models of Airway Inflammation

Emma M. Campbell and Nicholas W. Lukacs

1. Introduction

The suitability of any animal model of allergic airway inflammation is assessed by its ability to mimic human asthma. Thus, the ideal model would exhibit early and late antigen-induced airway responses, reversible bronchial hyperresponsiveness, peribronchial eosinophilia, and chronic lung remodeling ***(1)***. In this regard, the guinea pig has traditionally been the species of choice for many investigators and has been especially invaluable in elucidating mechanisms governing changes in airway physiology ***(2)***. However, in recent years, murine models of allergic airway disease have been extensively employed since the mouse can also be sensitized to a number of different antigens, developing an associated lung inflammatory response. This increased use of the mouse has largely been due to the commercial availability of species-specific reagents, as well as the development of gene knockout mice. In addition, the murine immune system has been relatively well characterized and in practical terms, the mouse is cost effective for experimentation.

These considerations have been important, not least, in the field of chemokine biology. Murine homologues to a number of human CC chemokines have been cloned, including MIP-1α, RANTES, MCP-1 (designated JE), MCP-3 (MARC), MCP-5, and eotaxin ***(3)***. However, a possible caveat concerning the mouse (in contrast to the guinea pig) is the apparent absence of the CXC chemokine, IL-8 ***(4)***. Nevertheless, murine (m) chemokine receptors CCR1-5 have also been identified and ligand selectivity has proved broadly similar to that for the human receptors. A notable exception is mMIP-1α binding to CCR3 in the mouse, unlike hMIP-1α which does not bind to hCCR3. It is worth noting that the rank affinities of the murine chemokines for their receptors do differ somewhat from the human counterparts ***(3)***.

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A number of murine models of allergic airway disease utilizing several different types of allergen and pharmaceutical challenge, have been successfully established. Sensitization to a range of allergenic proteins (for example alum-precipitated ovalbumin ***(2)*** and *Schistosoma mansoni* egg antigen ***(5)*** typically induce very similar inflammatory airway responses. This is characterized by an early lung neutrophilic infiltration, followed by a delayed eosinophilic infiltrate akin to the late phase reaction of human asthma ***(1)***. Associated with this is the presence of elevated levels of a limited panel of cytokines including IL-4 and IL-5, thus characterizing the response as one of a Th-2 phenotype ***(6,7)***. Attempts by a number of laboratories to assess the exact contribution that each of these cytokines makes to the development of allergic inflammation has often proved contradictory ***(7,8)***. This probably reflects differences in the strains of mice (*see* **Note 1**) as well as in the sensitization and challenge procedures used. For example, it has been suggested that in some murine models, the mast cell may function to augment responses induced by low levels of antigen, but is comparatively redundant when stronger procedures of immunization and challenge are used ***(9)***. Furthermore, few studies have focused on temporal changes in cytokine profiles following antigen challenge, in any one model. Our laboratory has utilized a parasite antigen-driven murine model to elucidate mechanisms of cytokine-induced allergic airway responses.

Sensitization and challenge using soluble antigen from the eggs of the helminthic parasite *S. mansoni* provides a useful model to study Th-2 allergic airway responses in the mouse. This model is somewhat unique in that the initial immunization involves injection of *S. mansoni* eggs from which the soluble antigen (SEA) is secreted over a prolonged period (0–7 d) *in situ*. This prolonged antigenic sensitization has been demonstrated to provide a preferential induction of a Th-2-type response (IL-4, IL-5) and associated eosinophilia, without the use of an adjuvant. The subsequent response is characterized by early increased levels of TNFα ***(9)*** peaking at between 1–8 h following SEA challenge and IL-4 ***(6)*** at 8–24 h. In this model, RANTES and MIP-1α have been identified as key eosinophil attractants ***(5)***. Furthermore, high serum SEA-specific IgE levels (10^4 to 10^5 titers) have also been detected in immunized mouse serum, as well as increased histamine levels in antigen-challenged mice, which are indicative of an allergic response.

2. Materials

2.1. Chemicals and Reagents

1. Pathogen-free mice (Charles River).
2. Peurto Rican strain of *Schistosoma mansoni* eggs (Biomedical Research Laboratory, NIH, Bethesda, MD).
3. Cockroach antigen (Bayer Pharmaceuticals, Etobicake, ON).

4. Incomplete Freunds adjuvant (Sigma, St. Louis, MO).
5. Methacholine (Sigma).
6. Ketamine (‘*Vetamine*,’ Bayer).
7. Methoxyflurane (‘*Metofane*,’ Mallinckrodt Veterinary Inc.).
8. Protease Inhibitors (Boehringer Mannheim, Mannheim, Germany).
9. Triton X-100.
10. Sterile 1.7% saline.
11. Phosphate-buffered saline (PBS) containing 25m*M* EDTA.
12. 4% paraformaldehyde.
13. Histological stains: Wright-Giesma, haematoxylin, and eosin.

2.2. Laboratory Equipment

1. Ultracentrifuge.
2. Stainless steel mesh (No. 100).
3. Nylon sieve—50 nm (Spectrum Medical Inc.).
4. Waring blender (Waring Commercial, New Hartford, CN).
5. Tissue tearor (Biospec Products, Racine, WI).
6. Whole body plethysmography apparatus (Buxco, Hartford, CN).
7. Respiratory pump ventilator (Harvard Apparatus).
8. 27-gauge butterfly and catheter (Abbot Laboratories, North Chicago, IL) for tail vein injections.
9. 18-gauge tube for tracheal catheterization.
10. Cytospin apparatus and microscope slides (Shandon, Runcorn, UK).
11. Apparatus for the preparation of histological sections.
12. Microscope (X1000 magnification).
13. 1-mL syringes.

3. Methods

3.1. Isolation of S. Mansoni Eggs and Soluble Egg Antigen (SEA) Preparation

1. Five- to eight-week-old mice are infected by percutaneous tail injection with 25–30 Puerto Rican strain of *S. mansoni* cercariae.
2. Heavily infected mice are killed and the livers containing the *S. mansoni* eggs are aseptically removed and placed in sterile 1.7% saline for 72 h at 4°C. The livers are washed with sterile saline and homogenized in a Waring blender (Waring Commercial) at low speed.
3. The homogenate is passed through a No. 100 stainless steel mesh to remove cell debris and the liver granulomas. The eggs are collected on a 50-nm nylon sieve (Spectrum Medical Inc.), recovered by rinsing into a 50-mL plastic centrifuge tube and centrifuged at 150*g* for 5 min.
4. The eggs are washed in 1.7 % saline and counted, before injection (*see* **Subheading 2.2.**).
5. Some eggs are used for the extraction of soluble egg antigen (SEA). The eggs are

ground on ice and the preparation spun in an ultracentrifuge at 100,000*g* for 2 h. The antigen is contained in the supernatant.

3.2. Sensitization and Induction of the Airway Response

1. CBA/J mice are immunized with 5000 isolated *S. mansoni* eggs intraperitoneally, at days 0 and 7 of the protocol.
2. On day 14, the mice are given an intranasal challenge (*see* **Note 2**) of 10 μg of the extracted SEA in 10 μL of PBS to localize the response to the airway.
3. Mice were rechallenged 6 d later by intratracheal administration (*see* **Note 3**) of 10 μg of SEA in 25 μL of sterile PBS or with PBS alone (vehicle). Responses are analyzed at various time points following antigen challenge.
4. Sensitized, vehicle-challenged animals are used throughout as time-matched controls.

Interestingly, we have more recently adapted this sensitization and challenge protocol to develop a murine model of cockroach antigen-induced allergic airway disease. Since 60% of inner city asthmatics have highly elevated IgE levels specific for cockroach antigens ***(11)***, this model will have clinical relevance. Mice (*see* **Note 1**) are immunized with 10 μg of cockroach allergen (Bayer Pharmaceuticals) in incomplete Freunds adjuvant on day 0. On day 14 the mice are given an intranasal challenge of 10 μg of cockroach allergen in 10 μL of diluent to localize the response to the airway. Mice are then rechallenged 6 d later by intratracheal administration of 10 μg of cockroach allergen in 50 μL of sterile saline or with saline alone (vehicle). Using the outlined procedure, a peribronchial eosinophilia is evident by 8 h post challenge, and peaking at 48 h with a concurrent impairment of lung function at these times. A similar time course of cytokine generation is observed as for SEA challenged animals.

3.3. Experimental Markers of Allergic Airway Inflammation

3.3.1. Measurement of Airway Hyperreactivity

Assessment of changes in airway physiology in allergic animals is arguably the most clinically relevant marker of the disease. Typically, this is determined by the observation of a hyperreactive response following administration of a bronchoconstrictor agent, such as methacholine. In recent years, such measurements in the mouse have been possible through the development of whole body plethysmographs (Buxco) specifically designed for low tidal volumes ***(12)***.

Using our models and specific sensitization protocols, we have consistently observed at least fivefold increases in airway hyperreactivity upon single allergen challenge (**Fig. 1**). This level of increase allows our laboratory to investigate mediators that may influence the airway response. Specifically, we have

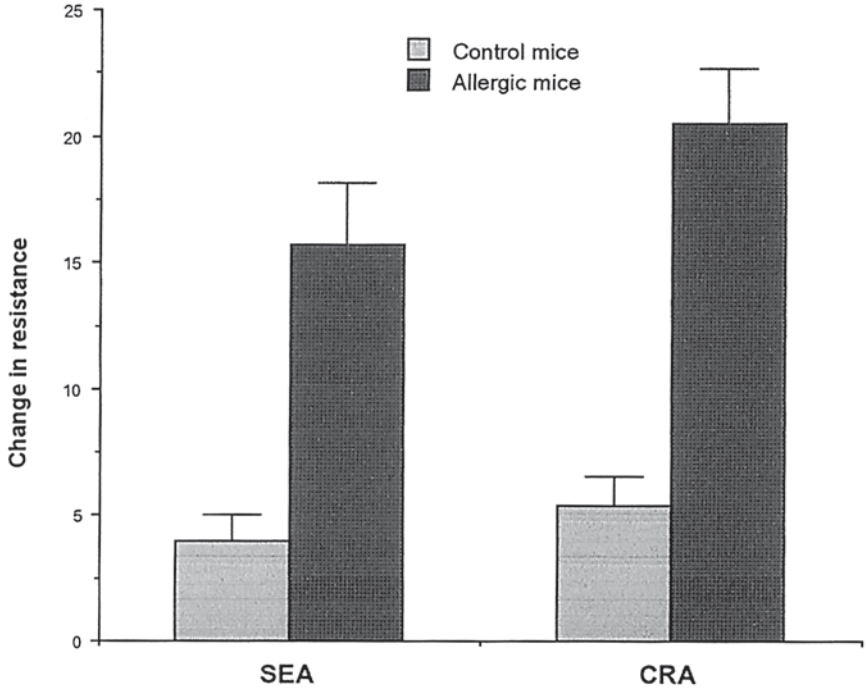

Fig. 1. Changes in airway resistance (cm of H_2O/mm/s) in allergic mice following methacholine administration. CBA/J mice were sensitized with either *Schistosoma mansoni* egg antigen (SEA) or cockroach antigen (CRA) and lung function monitored at 24 h postchallenge with the respective antigen. Mice were compared to sensitized, vehicle-challenged controls. Data represents mean ± SE of changes in resistance from at least four mice per group.

found that MCP-1 (JE) contributes toward hyperresponsiveness possibly through an ability to induce mast cell degranulation.

1. Mice are anesthetized with sodium pentobarbital (65 mg/kg administered intraperitoneally).
2. The animals are intubated via cannulation of the trachea with an 18-gage metal tube and ventilated (Harvard pump ventilator) at a tidal volume of 0.4 mL, frequency 120 breaths/min and positive end-expiratory pressure 2.5–3.0 cm H_2O.
3. The plethysmograph is sealed and the mouse ventilated for 5 min with readings monitored by computer. The accepted index of airway function is airway resistance, calculated by a change in pressure/a change in flow (*see* **Note 4**).
4. Once baseline levels are stabilized and initial readings are taken, a methacholine challenge is given via cannulation of the tail vein using a 27-gage needle for

injection. After determining a dose response curve (0.01–0.5 mg), an optimal dose is chosen (0.1 mg of methacholine) and used throughout the rest of the experiment. Following methacholine challenge, the response is monitored and the peak airway resistance recorded as a measure of airway hyperreactivity.

3.3.2. Quantitation and Differentiation of Leukocyte Populations in BAL and Lungs

1. Analysis of BAL: To assess migration of eosinophils into the airway, mice are subjected to a 1 mL bronchoalveolar lavage (BAL) with PBS containing 25 m*M* EDTA at various time points post-challenge. The cells are then dispersed using a cytospin (Shandon) and differentially stained with Wright-Giemsa stain. The cell types (mononuclear phagocytes, lymphocytes, neutrophils, and eosinophils) are expressed as a percentage based on 200 total cells counted per sample.
2. Histological analysis of the lung: The lungs are flushed with 1 mL of 4% paraformaldehyde, prior to removal. They are then stored in 4% paraformaldehyde overnight before embedding in paraffin. Multiple 50 μm sections are cut and differentially stained with Wright-Giemsa for the identification of eosinophils and viewed at 1000×. The individual eosinophils are counted from 100 high powered fields (HPF) per lung at each time point using multiple step sections of lung. Only eosinophils in the peribronchial region are counted, ensuring the enumeration of only those eosinophils within or immediately adjacent to an airway. The inflammation observed in these models is completely associated with the airway with little or no alveolitis.

3.3.3. Measurement of Chemokine Levels in the Lung

1. Measurement of chemokine proteins by specific ELISA. Chemokine protein from whole lung tissue can readily be detected using specific ELISAs ***(13)***. Isolated lung tissue is homogenized on-ice using a tissue tearor for 30 s in 1 mL of PBS containing 0.05% Triton-X100. The resulting supernatant is isolated after a high-speed spin (10,000*g*) and filtration through a 1.2-μm syringe filter. The protein is stable in the presence of protease inhibitor (Boehringer Mannheim) and storage at –20°C prior to use. Low, but detectable levels of chemokines can also be found in BAL fluid following the lavage protocol as given above.
2. Measurement of chemokine mRNA in the lung. Detailed methodological descriptions of these techniques are out of the scope of this chapter, suffice to say that a number of techniques are suitable to detect differences in chemokine message in whole lung tissue between treatment groups. Northern blot analysis has been used widely in the analysis of chemokine mRNA expression. Although mRNA levels can directly be assessed, this method can be time consuming and, in the case of whole tissue can be nonsensitive. In comparison, reverse transcriptase polymerase chain reaction (RT-PCR) is time effective and, providing the DNA amplifying primers are carefully selected, is very sensitive. However, since the amounts of amplified reverse transcribed DNA (and not RNA directly) are assessed, some caution should be exercised in terms of its quantitation.

4. Notes

1. Differences in immunology between mice strains have been widely documented. The authors of this review chapter have performed comparable studies using the same sensitization and challenge procedures in both CBA/J and C57B/6 mouse strains. Allergic CBA/J mice demonstrate a strong hyperreactive airway response to methacholine and contain greater levels of histamine (a marker of mast cell activation) in their BAL than C57B/6 mice. The C57B/6 mouse is somewhat resistant to the induction of mast cell-dependent airway hyperresponsiveness, possibly due to impairment of mast cell function ***(14)*** and has been demonstrated to produce lower levels of IL-4 during the priming of CD4+ T cells, compared to other stains ***(15)***. However, in contrast, the C57B/6 mice develop a far more pronounced eosinophilic pulmonary inflammation at later time points.
2. Intranasal administration of the antigen allows a local sensitization to develop. This initial intranasal challenge induces little cellular infiltration into the lungs of the mice upon histological examination.
3. A number of small animal models have administered the allergen by passive inhalation using a 1% aerosol. However, since the mouse is an obligate nose breather, calculation of the amount of antigen that reaches the lung is difficult since much is absorbed by the mucous membranes of the upper airways, or swallowed. However, this method does have the advantage of being performed on the conscious animal and is clearly noninvasive. More efficient delivery of antigen to the lung can be achieved by instillation directly into the trachea. Anesthesia for this recoverative procedure is 160 mg/kg ketamine (Vetamine, Bayer) intraperitoneally and inhalation of methoxyflurane (Metofane, Mallinckrodt Veterinary Inc.), which allows for a more precise and reproducible model of airway inflammation for the examination of specific mediator molecules.
4. Since the box is a closed system, a change in lung vol is represented by a change in box pressure (Pbox), measured by a differential transducer. The system was calibrated with a syringe that delivered a known vol of 2 mL. A second transducer was used to measure the pressure swings at the opening of the tracheal tube (Paw), referenced to the body box (i.e., pleural pressure), and to provide a measure of transpulmonary pressure (Ptp = Paw–Pbox). The trachea transducer was calibrated at a constant pressure of 20 cm H_2O. Resistance is calculated by the Buxco software by dividing the change in pressure (Ptp) by the change in flow (F) (dPtp/dF; units = cmH_2O/mL/s) at two time points from the vol curve based upon a percentage of the inspiratory vol.

References

1. Corrigan, C. J. and Kay, A. B. (1992) T cells and eosinophils in the pathogenesis of asthma. *Immunol. Today* **13,** 501–507.
2. Hudson, P. A., Church, M. K., Clay, T. P., Miller, P., and Holgate, S. T. (1988) Early and late phase bronchoconstriction after allergen challenge of nonanesthetized guinea pigs. *Am. Rev. Respir. Dis.* **137,** 548–557.

3. Premack, B. A. and Schall, T. J. (1996) Chemokine receptors: gateways to inflammation and infection. *Nature Med.* **2,** 1174–1178.
4. Yoshimura, T. (1993) cDNA cloning and expression of guinea pig neutrophil attractant protein-1 (NAP-1): NAP-1 is highly conserved in the guinea pig. *J. Immunol.* **151,** 6225–6236.
5. Lukacs, N. W., Strieter, R. M., Warmington, K., Lincoln P., Chensue, S. W., and Kunkel, S. L. (1997). Differential recruitment of leukocyte populations and alteration of airway hyperreactivity by C-C family chemokines in murine allergic airway inflammation. *J. Immunol.* **158,** 4398–4404.
6. Lukacs, N. W., Strieter, R. M., Chensue, S. W., and Kunkel, S. L. (1994) Interleukin-4-dependent pulmonary eosinophil infiltration in a murine model of asthma. *Am. J. Respir. Cell. Mol. Biol.* **10,** 526–532.
7. Foster, P. S., Hogan, S. P., Ramsay, A. J., Matthaei, K. I., and Young, I. G. (1995) IL-5-deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J. Exp. Med.* **183,** 195–201.
8. Corry, D. B., Folkesson, H. G., Warnock, M. L., Earle, D. J., Matthay, M. A., Wiener-Kronish, J. P., and Locksley, R. C. (1995) Interleukin 4 but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J. Exp. Med.* **183,** 109–117.
9. Galli, S. J. (1997) Complexity and redundancy in the pathogenesis of asthma: Reassessing the roles of mast cells and T cells. *J. Exp. Med.* **183(3),** 343–347.
10. Lukacs, N. W., Strieter, R. M., Chensue, S. W., Widmer, M., and Kunkel, S. L. (1995) TNFα mediates recruitment of neutrophils and eosinophils during airway inflammation. *J. Immunol.* **154,** 5411–5417.
11. Kang, B. C., Vellody, D., Homburger, H., and Yuninger, J. W. (1979) Cockroach cause of allergic asthma. *J. Allergy Clin. Immunol.* **63(2),** 80–86.
12. Martin, T. R., Gerard, R. P., Galli, S. J., and Drazen, J. M. (1988) Pulmonary responses to bronchoconstrictor agonists in the mouse. *J. Appl. Physiol.* **64,** 2318–2323.
13. Evanoff, H., Burdick, M. D., Moore, S. A., Kunkel, S. L., and Strieter, R. M. (1992) A sensitive ELISA for the detection of human monocyte chemoattractant protein-1 (MCP-1). *Immunol. Invest.* **21,** 39–42.
14. Ghildyal, N., Friend, D. S., Freelund, R., Austen, K. F., McNeil, H. P., Schiller, V., and Stevens, R. L. (1994). Lack of expression of the tryptase mouse mast cell protease 7 in mast cells of the C57BL/6 mouse. *J. Immunol.* **153,** 2624–2630.
15. Hsieh, C.-S., Macatonis, S. E., O'Garra, A., and Murphy, K. M. (1995) T cell genetic background determines default T helper phenotype development in vitro. *J. Exp. Med.* **181,** 713–721.

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Rat Models of Respiratory Inflammation

Andrew S. McWilliam and Patrick G. Holt

1. Introduction

In all medical and biological research, the ultimate aim is to understand pathological processes and to apply this understanding in the alleviation of human disease. Although laboratory-based molecular biology techniques are playing an ever increasing role, it is still necessary to use animal models of infection and disease. To date, this remains the only means by which we can obtain a contextual understanding of these processes. Since it is rare to find an animal model of disease that replicates human disease exactly, it is essential to choose the species and model to be investigated with great care. Although there are a great number of animal models of infection and disease that have been described in the scientific literature, there are still many practical constraints such as the availability of reagents, which may influence our choice of species or model.

The rat has been used extensively to study a range of biological and pathological processes and these have been reviewed in an excellent recent article ***(1)***. In particular, the rat offers many advantages as a model for respiratory diseases not the least being their size, which allows certain techniques such as intratracheal inoculation to be performed with greater ease, and also means that larger amounts of tissue and fluids are available. In addition, there is an extensive literature regarding inflammatory and immune responses in the rat. Disadvantages include the relative paucity of reagents such as monoclonal antibodies (MAbs) specific for rat antigens and the lack of transgenic and knockout rats. However, this situation is improving rapidly and many more rat-specific reagents are becoming available.

In the following sections we will describe the reagents and methodology for setting up and examining two models of respiratory inflammation in the rat. The first is a live Sendai virus model and the second is a model of acute bacte-

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rial inflammation using heat-killed *Moraxella catarrhalis*. These models are particularly well-suited to the rat and are excellent for studying inflammatory and immune reactions in the rat respiratory tract. In particular, Sendai infection in the rat has been used extensively and there are many publications describing the sequelae of infection ***(2–6)***.

2. Materials

2.1. Sendai Virus

1. Fertile chicken eggs day 9 or 10 after fertilization.
2. 37°C incubator.
3. Strong point source of light.
4. Soft-lead pencil.
5. Dentist or craft drill.
6. 26-gauge needle and 1 μL syringe.
7. Virus stock and ice bucket.
8. Sticky tape or paraffin wax.
9. 70% (v/v) ethanol.
10. Sterile saline.
11. Small pair of forceps.
12. Harvesting pipet. The end of a long glass pasteur pipet is sealed in a bunsen flame. The pipet is then held above the small pilot flame of a bunsen burner such that a small area approximately 1 cm from the tip of the pipet is heated. While holding the pipet above the flame blow gently into the large open end of the pipet until a small opening appears in the area being heated. Move the pipet further into the flame such that a section of pipet approximately 2–2.5 cm is melted and falls at a right angle to the rest of the pipet such that the hole is in the upper part of the bent section. Approximately 3–4 of these will be needed per dozen eggs to be harvested. Once made, these pipets are autoclaved.
13. Surgical or laboratory gloves, face mask, and gowns.

2.2. Moraxella catarrhalis

1. Blood agar plates and 37°C incubator.
2. Mueller-Hinton broth (Oxoid). This is prepared by dissolving 21 g of powder in one litre of distilled water and autoclaving at 121°C for 15 min. Alternatively, smaller amounts can be prepared and filter sterilized.

3. Methods

3.1. Preparation of Live Sendai Virus

3.1.1. Storage of Virus

1. To maintain maximum infectivity of Sendai virus it is essential that the virus be stored and handled properly. The virus is best stored either in liquid nitrogen or at –80°C. Under these conditions, the virus will remain viable for several years.

2. Before use, frozen virus should be allowed to thaw while packed in ice and used immediately after thawing. At all stages of handling, virus-containing samples should be kept on ice.

3.1.2. Growth of Virus

1. To obtain large amounts of virus for either cell fusion or infectious work, Sendai virus is best grown in the allantoic cavity of fertile chicken eggs. In this case, the choice of eggs is absolutely critical.
2. Fertile eggs should be obtained from a reputable chicken hatchery. Since some eggs from commercial sources may be contaminated with bacteria, diplomatic enquiries as to the microbial status of the eggs may be perspicacious.
3. The eggs should be ordered well in advance and should be day 9 or 10 after fertilisation when received. Transport of the eggs is important as even brief periods of cooling may alter viability and egg boxes should be well-wrapped in layers of newspaper and placed in polystyrene containers for transport.
4. Once in the laboratory, the egg boxes, still wrapped in newspaper, are placed in an incubator at 37°C.
5. While the stock virus is thawing on ice, the eggs may be candled. This is a process whereby the site of inoculation of the virus is chosen.

3.1.3. Fertile Egg Candling

1. This is best performed in a darkened room and in our experience is made easier if white or pale colored eggs are used and no more than one dozen eggs are handled at one time. For this procedure a strong light source is necessary. This may be a small powerful torch but a flexible fiber optic light source of the sort commonly found on dissecting microscopes is ideal. The light is placed in contact with the egg to illuminate the internal contents of the egg. The embryo will be readily apparent as are the blood vessels.
2. After examination, an area is chosen which is as far away from both the embryo and blood vessels as possible. This will be the site of inoculation so it is important not inject into a blood vessel as this will result in the death of the embryo. Using a soft-lead pencil this location is marked on the surface of the egg.
3. While illuminated, the air pocket that is visible at the larger domed end of the egg is circumscribed with the pencil.
4. Once candling is complete, the eggs are immediately placed in the incubator because it is important that their temperature is not allowed to drop.

3.1.4. Inoculation of Eggs

1. This procedure is best performed in a laminar flow hood but with care can easily be done on the laboratory bench. Although Sendai is a rodent virus, it is always a sensible precaution for laboratory personnel to wear gloves, gowns and masks when handling the virus or infected animals.
2. Once thawed, stock virus is diluted approximately 1/100 with sterile saline and this dilution is kept on ice.

3. The pencil marked site is briefly swabbed with 70% ethanol. Note that excess ethanol may be absorbed by the egg and cause damage.
4. Using either a dentist's drill or a small craft drill with a burred bit, the outer layer of shell is gently removed until either the inner white membrane is apparent or the shell is thin enough to penetrate with a needle. This operation requires a degree of finesse and it would be well to practice on a spare egg.
5. Approximately 100 μL of diluted stock virus is injected slowly through the allantoic membrane. If a small amount of blood appears this may be ignored. If the solution blebs slightly onto the surface of the egg, wait for a few seconds and this will generally reabsorb.
6. The site of injection must now be sealed and this can be done with either a small drop of melted paraffin wax or, more easily, with adhesive tape.

3.1.5. Incubation of Inoculated Eggs

Specifically designed egg incubators are available but are not usually necessary. The inoculated eggs should be incubated with the pointed end downwards at 37°C for 2–3 d. During this time the eggs may be turned slightly, but we have not found this absolutely necessary.

3.1.6. Harvesting of Allantoic Fluid

1. After incubation, the eggs are placed at 4°C for at least 2 h to allow the eggs to cool, which kills the embryo and allows the blood to coagulate to reduce possible adsorption of virus to the red cells. This reduces the amount of blood which may contaminate the allantoic fluid during harvesting.
2. Sterilize the blunt end of the egg with 70% ethanol and use the forceps (sterilized in 70% ethanol) to remove the shell from above the airspace, which was marked with pencil as described in **Subheading 3.1.3., step 3**. This will expose the chorioallantoic membrane, however, care should be taken not to remove too much shell as this will tear into the membrane.
3. Using the sterile forceps, tear the membrane and push it to one side of the egg. Insert the glass pasteur harvesting pipet described in **Subheading 2.1.2.**, into the allantoic fluid and with gentle suction remove the allantoic fluid. The fluid should enter the small hole in the pipet while the bent section prevents the membrane or other contaminants from being sucked in and blocking the hole. Each egg should produce around 5–10 μL of fluid.
4. If there is a significant amount of blood in the fluid or if the fluid appears cloudy or odorous then discard the entire egg. Use a new pipet every 3–4 eggs and place the collected allantoic fluid into a sterile glass vessel, which is kept on ice.
5. If the allantoic fluid is to be used to establish a viral infection, then allantoic fluid should be collected from uninfected eggs and this may then be used as a control inoculum.
6. Once harvested, the allantoic fluid should be clarified by centrifugation at 3000*g* for 20 min at 4°C. It may then be aliquoted into appropriate volumes and stored at –80°C or in liquid nitrogen until required.

7. A sample of virus should be retained and tested by hemagglutination on chicken or duck red blood cells. An excellent description of this technique has been published *(7)*.

3.1.7. Animal Inoculation

1. Rat strains vary in susceptibility to Sendai virus; however, in general the Brown Norway strain has been most extensively used and gives good respiratory inflammatory responses *(5,8)*.
2. It is a good idea to have the animals to be inoculated tested for serological evidence of Sendai prior to planning a series of experiments (*see* **Note 1**).
3. If nasal inoculation is to be performed then the animals must first be anaesthetized by intraperitoneal injection of chloral hydrate. For this, a 5.7% (w/v) solution is prepared in phosphate-buffered saline (PBS) pH 7.4, and 250 μL injected per 50 g of body weight.
4. Once anesthetized, 50 μL of allantoic fluid, containing approximately 10^3 hemagglutinating units of virus, is inoculated slowly into the external nares. (*see* **Note 2**). This should be done slowly and in small amounts to allow time for the animal to inhale the solution. If it is administered too fast, there is a risk of either drowning a small animal or having it sneezed out. This is ideally performed using a Gilson pipet fitted with a long plastic sequencing tip.
5. While the animal is anesthetized, it is important to keep it warm to prevent hypothermia.
6. Animals inoculated with sterile allantoic fluid should not be housed with Sendai infected animals to prevent cross infection.
7. Sendai virus can be detected within the respiratory tract epithelial lining by immunohistochemistry within 2 d after inoculation and will have cleared by 7–10 d. The peak of the inflammatory response occurs at day 5 postinoculation *(8)*.

3.2. *Heat-Killed* Moraxella catarrhalis *Bacteria*

3.2.1. Preparation of Bacteria

1. *Moraxella catarrhalis* is also known in the literature as *Branhamella catarrhalis* and type strains are available from the ATCC or clinical microbiology laboratories may provide clinical isolates. It is strongly recommended that a biohazard safety hood is used when handling large quantities of the bacterium (*see* **Note 3**).
2. The bacterium is grown on blood agar plates and stored in a mixture of Mueller-Hinton broth containing 20% (v/v) glycerol. In this mixture, bacteria can be stored in small aliquots for several years at –70°C.
3. To prepare plate cultures, frozen stocks are placed on dry ice and are "stabbed" with a hot wire loop. Material adhering to the loop is then plated onto the blood agar plates. Frozen stocks may require several passages to regain growth characteristics.
4. On agar plates, colonies of *M. catarrhalis* can be easily identified by their capacity to be moved across the surface of the agar with a sterile loop.

5. If larger culture volumes are required then 2.5-L Ehrlenmeyer flasks containing a maximum of one liter of Mueller-Hinton broth are prepared and autoclaved. Once cooled, this broth may be seeded directly with colonies from the agar plate; however, this often results in poor yields and slow growth. It is better to seed this volume of culture with a small amount of overnight broth culture.
6. To prepare a seeding culture, fill a 25 μL universal with approximately 10 μL of sterile Mueller-Hinton broth and seed these with 1–2 colonies from the blood agar plate. This is then incubated at 37°C overnight with vigorous shaking at approximately 200 rpm.
7. Ehrlenmeyer flasks may then be seeded with 5–10 μL of this culture and are again incubated for 24–36 h at 37°C with vigorous shaking.
8. The liquid culture may then be harvested by centrifugation at 3000–4000 rpm for 15–20 min. If the bacterial pellet is to be resuspended, then it is a good idea to gently break up the pellet with a sterile glass rod and resuspend slowly by adding small volumes of diluent and mixing as one does in preparing a roux for white sauce in the kitchen. All supernatants should be mixed with concentrated bleach or autoclaved before disposal, and centrifuge tubes soaked in 70% (v/v) ethanol.
9. The pellet should be washed several times in either sterile PBS or sterile distilled water. For aerosolization, the pellet from 500 μL of broth culture is resuspended in 50 μL of distilled water and frozen at –70°C in 10 μL aliquots.
10. Before use, frozen aliquots should be allowed to thaw overnight at 4°C and may then be killed by heating at 60°C for 30–40 min with regular mixing during this time. It is recommended that viability tests be performed after this heat treatment to ensure adequate killing.

3.2.2. Aerosol Administration of M. catarrhalis

1. *M. catarrhalis* has a propensity to form clumps, so if the bacteria is to be placed in an aerosol machine or nebulizer, the preparation must first be briefly sonicated or alternatively passed vigorously several times through a 26-gauge needle to break up these clumps (*see* **Note 4**).
2. We routinely perform aerosolization experiments using a Tri-R aerosol apparatus produced by the Glas-Col company of Terre Haute, IN, USA, however, the bacteria may also be administered by direct intratracheal installation.

4. Notes

1. It should be noted that Sendai virus is extremely infectious for rodents and very difficult to eradicate from a rodent housing facility once established. Animals infected with Sendai should not be housed in the same facility as other rodents used for experimental purposes and personnel handling. Sendai should be restricted from entering any rodent housing facility. Appropriate precautions should also be taken when disposing of virus-contaminated material.
2. Sendai may be administered either by aerosol or by direct inoculation into the nose.

3. It should always be kept in mind that *M. catarrhalis* is a human pathogen and appropriate precautions should be taken when growing, handling, and aerosolizing this organism.
4. The amount of suspension and the time of aerosol are totally discretionary. Neutrophils will begin to appear in the bronchoalveolar lavage fluid within 30 min of aerosol ***(9,10)*** and this may be used as a guide to the effectiveness of the preparation or of the aerosolization. There is also a significant variation in the degree of response seen depending on the rat strain used. Similarly, decreased neutrophil responses are seen in younger animals.

References

1. Harroff, H. H. (1997) Use of animal models in pediatric research, in *Advances in Pediatrics*, Vol. 44, Mosby-Year Book Inc., St. Louis, MO, pp. 453–489.
2. Carding, S. W. Allan, W., Keyes, S., Hayday, A. Bottomly, K., and Doherty, P. (1990) Late dominance of the inflammatory process in murine influenza by g/d^+ T cells. *J. Exp. Med.* **172,** 1225–1231.
3. Castleman, W. L. (1983) Respiratory tract lesions in weanling outbred rats infected with Sendai virus. *Am. J. Vet. Res.* **44,** 1024–1031.
4. Castleman, W. L. (1984) Alterations in pulmonary ultrastructure and morphometric parameters induced by parainfluenza (Sendai) virus in rats during postnatal growth. *Am. J. Pathol.* **114,** 322–335.
5. Castleman, W. L., Brundage-Anguish, L. J., Kteitzer, L., and Neuenschwander, S. B. (1987) Pathogenesis of bronchiolitis and pneumonia induced in neonatal and weanling rats by parainfluenza (Sendai) virus. *Am. J. Pathol.* **129,** 227–286.
6. Castleman, W. L., Northrop, P. J., and McAllister, P. K. (1989) Replication of parainfluenza (Sendai) virus in isolated rat pulmonary type II alveolar epithelial cells. *Am. J. Pathol.* **134,** 1135–1142.
7. Barrett, T. and Inglis, S. C. (1985) Growth, purification and titration of influenza viruses, in *Virology: A Practical Approach* (Mahy, B. W. J., ed.), IRL Press, Oxford, UK, pp. 119–150.
8. McWilliam, A. S., Marsh, A. M., and Holt, P. G. (1997) Inflammatory infiltration of the upper airway epithelium during Sendai virus infection: involvement of epithelial dendritic cells. *J. Virol.* **71,** 226–236.
9. McWilliam, A. S., Nelson, D., Thomas, J. A., and Holt, P. G. (1994) Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces. *J. Exp. Med.* **179,** 1331–1336.
10. McWilliam, A. S., Napoli, S., Marsh, A. M., Pemper, F. L., Nelson, D. J., Pimm, C. L,. Stumbles, P. A., Wells, T. N. C., and Holt, P. G. (1996) Dendritic Cells are recruited into the airway epithelium during the inflammatory response to a broad spectrum of stimuli. *J. Exp. Med.* **184,** 2429–2432.

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Murine Model of Crescentic Nephritis

Clare Lloyd and Jose-Carlos Gutierrez-Ramos

1. Introduction

In humans rapidly progressive glomerulonephritis is characterized by glomerular inflammation and the formation of glomerular crescents, composed of an infiltrate of mononuclear inflammatory cells and proliferating parietal epithelial cells. As disease progresses the crescents infringe on the urinary space, compressing the glomerular tuft, ultimately causing nonreversible acute renal failure. This process is almost always associated with severe interstitial and periglomerular inflammation. Typically, the inflammatory infiltrate gives way to a progressive fibrotic process involving the crescents and the periglomerular and peritubular interstitium, accompanied by tubular atrophy and progressive renal failure *(1)*.

Although the pathogenesis of crescentic glomerulonephritis is incompletely understood and likely involves several convergent pathways, there is general agreement that the infiltrating leukocytes play a central role *(2)*. A variety of in vivo and in vitro studies have defined a role for chemokines in eliciting leukocyte migration to the kidney during the development of crescentic nephritis (reviewed in **refs.** *3,4*) using models of nephritis in rats, rabbits, and more recently mice. The model of crescentic nephritis described here is dose dependent and varies from mild glomerular hypercellularity to anuric renal failure and early death from extensive glomerular crescent formation. The control mice do not exhibit any histological changes. As early as 12 h after the first injection of nephrotoxic serum (NTS) proteinaceous casts are present in renal tubules, coincident with the onset of proteinuria. After 24 h, leukocytes are evident within the interstitium and by day 3 prominent perivascular infiltrates can be seen. At this point the glomeruli are clearly hypercellular. On day 5, some

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glomeruli contain crescents and interstitial and perivascular infiltrates are pronounced. By day seven, 55% of glomeruli exhibit prominent cellular crescents and marked abnormalities are present within the tubules and interstitium. These include severely dilated tubules with flattened or denuded epithelia, and expansion of the interstitium due to edema, interstitial infiltrates, and the onset of fibrosis (as determined by deposition of extracellular matrix). On day 14 the fibrotic process occurs diffusely throughout the interstitium and extends to involve some of the glomerular crescents, apparently by invasion through Bowman's capsule. This model shows many of the characteristic histological features of the human disease, which occur with a concomitant decline in renal function. Moreover, disease is relatively easy to induce, occurs with a high incidence, and is highly reproducible.

2. Materials

2.1. Induction of Nephritis

1. Sieves: 250 μ, 106 μ, 75 μ (VWR, Westchester, PA).
2. Normal sheep serum: technical grade; should be kept sterile and frozen.
3. Freund's complete adjuvant: (Pierce, Rockford, IL).
4. Nephrotoxic serum: should be kept frozen until used and then on ice until injected.

2.2. Analysis of Disease Development

1. Spectrophotometer.
2. Protein standards: Labtrol liquid chemistry control (Dade Diagnostics #B5100, VWR).
3. Standard histology equipment: tissue processor, microtome.
4. Routine stains: hematoxylin and eosin (both from VWR or other general chemical company).

3. Methods

3.1. Induction of Nephritis

3.1.1. Preparation of Nephrotoxic Serum

Nephrotoxic serum (NTS) is prepared by immunizing male sheep with a lysate of rat glomeruli, isolated by differential sieving. This procedure has been described previously in great detail ***(5)***.

1. Swab animals with alcohol, remove kidneys, place in Hanks.
2. Peel away capsule, cut slices around cortex.
3. Chop up slices in Hanks, then squash firmly with syringe plunger.
4. Pass through series of graded sieves, squirting buffer through with syringe until glomeruli sit on top of last sieve. Place in a beaker of buffer and glomeruli will float off.

5. Inspect microscopically. If tubular contamination is present repeat sieving or split into several tubes with buffer. Leave to settle, glomeruli will sink, so aspirate rest off. You should have passed about 500 mL of buffer through the sieves.
6. Very few glomeruli are needed, but they should be free from tubular contamination (they look very like cauliflower).
7. Glomerular lysate is used as an innoculum for generation of polyclonal sheep antibodies using a commercial service (e.g., Research Genetics, Huntsville, AL).
8. The ensuing sheep serum is heat-inactivated by incubation in a waterbath at 56°C for 30 min, absorbed with rat red blood cells and serum proteins, and filter-sterilized. For storage, the serum is lyophilized and frozen at –70°C. As reported elsewhere ***(6)***, NTS has moderate reactivity to type IV collagen and laminin, and substantial reactivity to glomerular cell membrane proteins, particularly β1 integrin and its accompanying α chain.

3.1.2. Induction of Crescentic Nephritis

The protocol for inducing crescentic glomerulonephritis in mice was adapted from an earlier study ***(7)***, modified for our NTS to avoid rapid lethality from acute renal failure while ensuring adequate tissue injury for analysis (*see* **Fig. 1**).

1. Preimmunze CD1 mice (weighing around 30 g) by a subcutaneous injection of 200 μg normal sheep IgG in Freund's complete adjuvant.
2. After 5 d inject mice intravenously with 50 μL NTS on three consecutive days. Inject controls with 50 μL normal sheep serum on the same schedule.
3. Collect urine overnight by placing mice in metabolic cages, prior to sacrifice. During this collection, allow mice free access to water but not food.
4. To analyze disease progression, sacrifice mice at various intervals from 12 h to 3 wk after the first dose of NTS. At the time of sacrifice, obtain serum by cardiac puncture.
5. For tissue collection, take samples according to the method of analysis: snap-freeze portions of kidney in liquid nitrogen for RNA extraction, roll tissue in Tissue Tek OCT (Cryoform, IEC, Needham, MA) and snap freeze in isopentane cooled over dry ice; fix tissue in 4% paraformaldehyde for *in situ* hybridization or in 10% buffered formalin for routine histology.

3.2. Analysis of Disease Development

3.2.1. Morphological Analysis

1. Fix kidney halves overnight at 4°C in 10% neutral buffered formalin (Fisher), embed in paraffin, and section at 3 μm.
2. Stain the sections with hematoxylin and eosin following standard techniques: dewax sections in xylene; hydrate through graded alcohols to water; stain for 4 min in Harris hematoxylin; wash well in running tap water until sections are "blue" (5 min or less); differentiate 5–10 s in 1% acid alcohol (1% HCL in

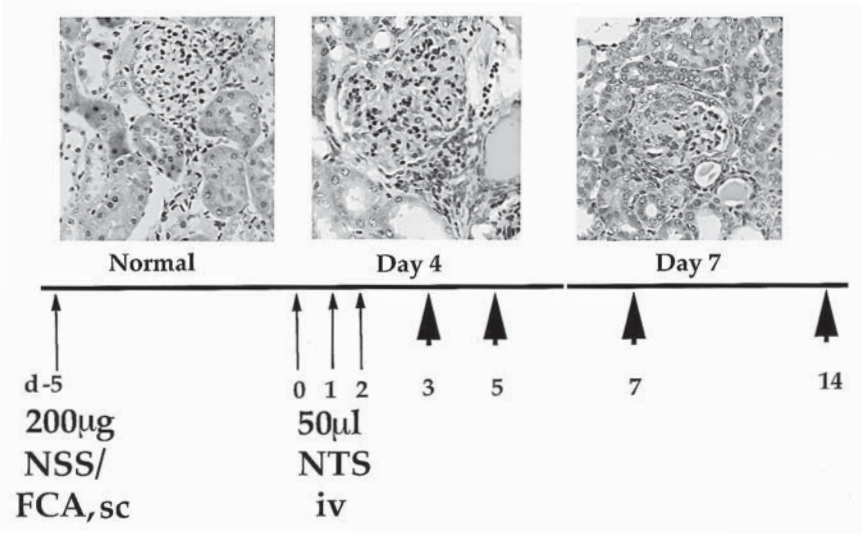

Fig. 1. Scheme for induction of murine crescentic nephritis. (NSS, normal sheep serum; NTS, nephrotoxic serum; sc, subcutaneous; iv, intravenous.)

70% ethanol); wash well in running tap water until sections are again "blue" (5 min or less); stain in 1% eosin Y for 10 min; wash well in running tap water for 1–5 min; dehydrate through alcohols, and clear in xylene before mounting using Permount (VWR) or similar medium.

3. Examine sections microscopically for evidence of glomerular hypercellularity and necrosis, mesangial thickening, formation of glomerular crescents, interstitial infiltrates, and development of glomerular and interstitial fibrosis, comparing NTS-treated mice to those treated with normal size and shape (NSS) mice (**Fig. 2**).
4. Obtain a crescent score by counting the percentage of glomeruli showing cellular crescents in at least 100 glomeruli per mouse.

3.2.2. Assessment of 24 h Proteinuria

Urine protein excretion should be measured on timed overnight specimens collected at intervals from 3 d to 3 wk from individual mice in metabolism cages and is assayed by the sulphosalicylic method.

1. Take two tubes for each urine sample, one for the sample, the other for the blank.
2. Add to each tube urine diluted with PBS (pH7.4) to a total volume of 1 mL.
3. To the sample tube, add 3 mL of sulphosalycilic acid (3% w/v in water) and 3 mL of PBS (pH 3.5) to the blank tube.
4. Let stand for 10 min before reading the OD at 650 nm.
5. Protein concentration is calculated by reading from a standard curve, prepared from Labtrol protein chemistry control reagent, diluting the Labtrol with SSA to reach a range of concentrations between 0.05 mg/mL and 2 mg/mL total protein.

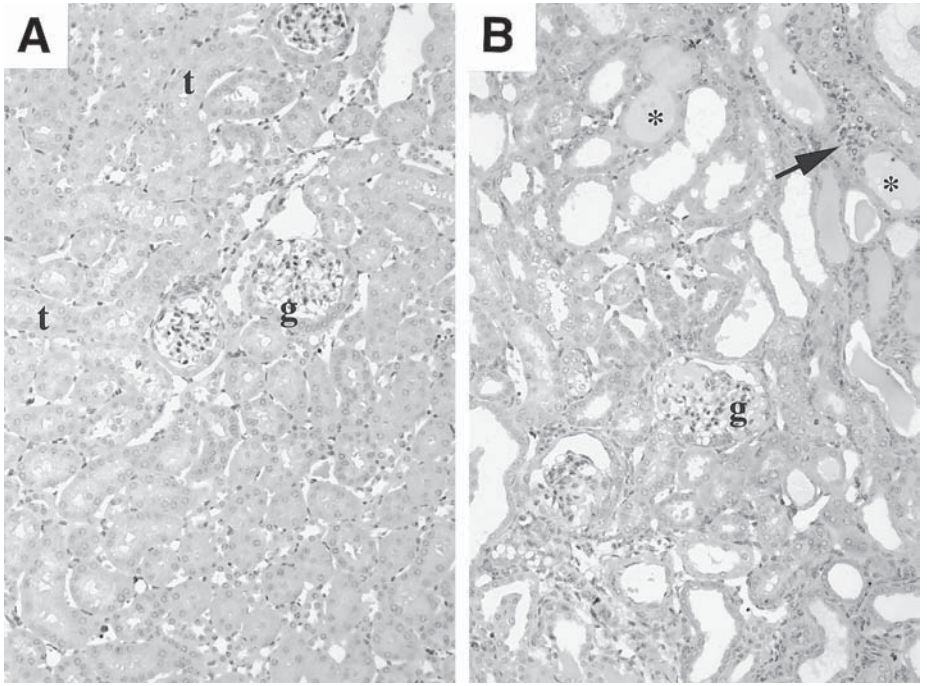

Fig. 2. Renal morphology in normal mice **(A)** compared to mice observed 7 days after induction of crescentic nephritis **(B)**. Changes in glomeruli (g) and tubules (t) include protein deposits and flattened epithelial (marked by *), cellular crescents in glomeruli (g) and the presence of interstitial inflammatory infiltrates (arrow).

6. Proteinuria is expressed as the total amount of excreted over a 24 h time period and is calculated:

$$\text{mg/24 h} = \text{mg/mL protein} \times \text{dilution} \times \text{vol} \times \frac{24}{T}$$

where:

mg/mL is the concentration obtained from the reading OD values from the standard curve

vol is the volume of urine obtained from the overnight collection

T (collection time) is the time span (in hours) over which urine was collected

3.3. Analysis of Chemokine Expression

3.3.1. Assessment of Chemokine mRNA Expression

1. Extract total RNA from kidneys isolated from NTS and NSS treated mice using the guanidinium thiocyanate/acid phenol procedure ***(8)***.

2. Prepare Northern blots ***(9)*** with 20 μg of total RNA, purified as indicated previously, and fractionate in a 1.5% agarose/formaldehyde gel before blotting onto a nylon membrane (Genescreen, DuPont).
3. Membranes were probed using ^{32}P-labeled probes for chemokines applied in 50% formamide hybridization solution at 42°C for 18 h. We have used: Eotaxin ***(10)***, MCP-5 ***(11)***, RANTES ***(12)***, MCP-1/JE ***(13)***, MIP-1α ***(14)***, and TCA-3 ***(15)***.
4. Wash blots in 2X SSC/1% SDS at 45°C and expose at –70°C on Kodak XAR5 film (Sigma).

An attractive alternative to Northern analysis is to measure expression by multiprobe RNAse protection. This is a highly sensitive and specific method which allows the simultaneous detection and quantification of several mRNA species in a single sample of total RNA. Thus, comparative analysis of different mRNA species within samples is possible and, by incorporating probes for housekeeping genes, the levels of RNAs can be compared between samples. Template sets for chemokines and their receptors (human and mouse) are available for performing multiprobe RNAs protection assays (Pharmingen, San Diego, CA).

3.3.2. Chemokine Protein Expression

We have determined localization of chemokines within tissue sections by using specific monoclonal (or polyclonal) antibodies. Many of these are available commercially from Genzyme (Boston, MA) or Pharmingen (San Diego, CA).

Although conditions for individual chemokines and tissues vary, we have used the following protocol to stain for a variety of chemokines ***(10,11,16)***.

General considerations:

- All incubations are carried out under humidified conditions;
- Wash = dip slides in dish of 0.1 *M* PBS supplemented with 0.2% gelatin (PBSG), then replace with fresh buffer and place on stirplate for 5 min.

1. 3μ frozen sections should be air-dried, fixed in 2% paraformaldehyde (5 min, 4°C) and methanol (10 min, –20°C). Both fixatives should be precooled. Paraffin sections should be taken through xylene and alcohols to water. Overlay sections with 20% fetal calf serum (FCS) in PBS for 15 min.
2. Incubate overnight at 4°C with polyclonal/monoclonal antibody (PAb; MAb) or normal rabbit serum (both diluted in PBS supplemented with 0.1% bovine serum albumin [BSA]).
3. Block endogenous peroxide by incubation for 20 min in methanol containing 0.3% hydrogen peroxide.
4. Block nonspecific staining due to cross reaction with endogenous avidin or biotin by incubation with avidin solution followed by biotin solution, both for 20 min (use Vectorstain kit, as directed, Vector Labs, Burlingame, CA).
5. Incubate with biotinylated secondary antibody diluted in 10% normal mouse serum PBS for 30 min.

6. Incubate with streptavidin peroxidase complex (Dako, as directed) for 30 min.
7. Flood with peroxidase substrate solution (400 mg diaminobenzidine in 10 mL PBS, containing 0.01% hydrogen peroxide or Vector kit) for 10 min.
8. Counterstain with haematoxylin, dehydrate, pass through xylene, mount in DPX.

4. Notes

1. Nephrotoxic serum should be titrated for each new preparation to assess disease severity. For each new batch inject groups of mice with NTS over a range of concentrations or volumes, and collect urine and kidney tissue on day 7. Compare degree of proteinuria and crescent score to ensure continuity between batches of NTS. We have chosen a dose of 50 μL of our current NTS, which induces between 50–60% crescent formation by day 7 in CD1 mice. An in-depth description of the production of NTS is provided in **ref *5***.
2. Disease development differs between strains of mice. Crescent formation is much slower in C57/B6 mice, with the same dose of NTS inducing 25% crescent formation by day 7.
3. Although the dose of NTS per mouse is 50 μL, we inject this in 100 μL of PBS for greater accuracy during intravenous injections.
4. Labtrol reagents differ in total protein concentration between lots. The total protein values can be found on the instruction sheet for each particular lot. Take an average value, excluding any high standard deviation (should be 69–74 mg/mL).
5. We have found that normal proteinuria values for our male CD1 mice vary, but we assume anything over 20 mg/24 h to be abnormal. However, it should be noted that overnight collection is absolutely essential.
6. Many commercial laboratories run renal profile tests that include proteinuria, urine creatinine, and serum urea nitrogen, as well as other clinical parameters pertinent to the development of renal dysfunction. Although these services tend to be expensive, they offer a fast and reliable way to measure a range of parameters.
7. RNA was extracted from PBS-treated mice at the same time points as NTS and NSS mice to determine basal levels of chemokines.
8. It is necessary to test concentrations for each new antibody, whether primary or secondary. For blocking before application of the primary, use serum from the same species that your secondary is made in e.g., if your secondary is made in a rabbit, block with normal rabbit serum but if your secondary is made in a pig use swine serum. For secondary antibodies it is advisable to use F(ab)' fragments (to minimize cross reaction with FcR on macrophages or B-cells).

References

1. Salant, D. J. (1987) Immunopathogenesis of crescentic glomerulonephritis and lung purpura. *Kidney Int.* **32,** 408–425.
2. Lloyd, C. M., Minto, A. W., Dorf, M. E., Proudfoot, A., Wells, T. N. C., Salant, D. J., and Gutierrez-Ramos, J.-C. (1997) RANTES and monocyte chemoattractant protein-1 (MCP-1) play an important role in the inflammatory phase of crescentic

nephritis but only MCP-1 is involved in crescent formation and interstitial fibrosis. *J. Exp. Med.* **185(7),** 1371–1380.
3. Schlondorff, D., Nelson, P. J., Lucknow, B., and Banas, B. (1997) Chemokines and renal disease. *Kidney Int.* **51,** 610–621.
4. Lloyd, C. M., and Gutierrez-Ramos, J. C. (1998) The role of chemokines in tissue inflammation and autoimmunity in renal diseases. *Curr. Opin. Nephrol. Hypertens.* **7,** 281–287.
5. Salant, D. J. and Cybulsky, A. V. (1988) Experimental glomerulonephritis. *Methods Enzymol.* **162,** 421–461.
6. O'Meara, Y. M., Natori, Y., Minto, A. W., Goldstein, D. J., Manning, E. J., and Salant, D. J. (1992) Nephrotoxic antiserum identifies a b1-integrin on rat glomerular epithelial cells. *Am. J. Physiol. Renal Fluid Electrolyte Physiol.* **262,** F1083–F1091.
7. Morley, A. R. and Wheeler, J. (1985) Cell proliferation within the Bowman's capsule in mice. *J. Pathol.* **145,** 315.
8. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159.
9. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
10. Gonzalo, J.-A., Jia, G.-Q., Aguirre, V., Friend, D., Coyle, A. J., Jenkins, N. A., et al. (1996) Mouse eotaxin expression parallels eosinophil accumulation during lung allergic inflammation but it is not restricted to a Th2-type response. *Immunity* **4,** 1–14.
11. Jia, G. Q., Gonzalo, J. A., Lloyd, C., Kremer, L., Lu, L., Martinez, C., Wershil, B. K., and GutierrezRamos, J. C. (1996) Distinct expression and function of the novel mouse chemokine monocyte chemotactic protein-5 in lung allergic. *J. Exp. Med.* **184,** 1939–1951.
12. Heeger, P., Wolf, G., Meyers, C., Sun, M. J., O'Farrell, S. C., Krensky, A. M., and Neilson, E. G. (1992) Isolation and characterization of cDNA from renal tubular epithelium encoding murine RANTES. *Kidney Int.* **41,** 220–226.
13. Rollins, B. J., Morrison, E. D., and Stiles, C. D. (1988) Cloning and expression of JE, a gene inducible by platelet-derived growth factor and whose product has cytokine-like properties. *Proc. Natl. Acad. Sci. USA* **85,** 3738–3742.
14. Widmer, U., Yang, S., van Deventers, S., Manogue, K. R., Sherry, B., and Cerami, A. (1991) Genomic structure of murine macrophage inflammatory protein-1 and conservation of potential regulatory sequences with a human homolog, LD78. *J. Immunol.* **146,** 4031–4040.
15. Burd, P. R., Freeman, G. J., Wilson, S. D., Berman, M., DeKruyff, R., Billings, P. R., and Dorf, M. E. (1987) Cloning and characterization of a novel T cell activation gene. *J. Immunol.* **139,** 3126.
16. Pan, Y., Lloyd, C., Zhou, H., Dolich, S., Deeds, J., Gonzalo, J.-A., et al. (1997) Neurotactin: a membrane-anchored chemokine upregulated in brain inflammation. *Nature* **387,** 611–617.

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Rabbit Models of Pneumonia, Peritoneal Sepsis, and Lung Injury

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1. Introduction

The Acute Respiratory Distress Syndrome (ARDS) is a form of lung injury characterized by the rapid onset of severe hypoxemia and diffuse pulmonary infiltrates, with normal cardiac filling pressures ***(1)***. The pathology of ARDS includes striking injury to the alveolar epithelium, and the accumulation of neutrophils (PMN), protein, and fibrin-rich exudates in the alveolar spaces of the lungs ***(2)***. In addition there is evidence of pulmonary microvascular injury, with intravascular microthrombi and endothelial damage ***(3)***. Sepsis and trauma are the two major clinical risk factors that precede ARDS, and account for about two-thirds of the cases in major centers ***(4)***. ARDS also occurs following aspiration of gastric contents, mixed drug overdoses, fresh and salt-water drowning, and other less common clinical events.

In humans, sepsis can follow pulmonary and nonpulmonary infections, and ARDS can occur in either clinical setting (reviewed in **ref. *5***). "Sepsis" is defined as the systemic response to a definite or probable tissue infection, and typically includes changes in body temperature, blood leukocyte counts, hemodynamic parameters, and organ function ***(6)***. There is an important distinction to be made between "bacteremia," defined simply by a positive blood culture, and sepsis, which includes a specific systemic response. Most cases of bacteremia are not associated with clinical evidence of sepsis, and bacteremia by itself is rarely associated with ARDS ***(7)***. Rather, it appears that a primary tissue infection is an important requirement for the onset of sepsis in humans, and an important antecedent for ARDS whether or not bacteremia is detectable ***(8)***.

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In order to understand the pathogenetic mechanisms that link sepsis with ARDS and the failure of other organ systems, experiments have been conducted with a range of animal models, using a variety of technical methods and organisms to induce sepsis ***(9,10)***. Mice and rats have advantages, as many animals can be studied at the same time, and a variety of immunological reagents are available. In addition, the availability of transgenic mice has made it possible to test the importance of specific pathways. However, mice and rats differ in important ways from humans. In particular, their chemokine systems are different from those of humans and their nitric oxide pathways, particularly within macrophages are significantly more active. Rabbits have advantages, as they are large enough to permit physiological studies, and their chemokine systems and nitric oxide pathways are similar to those of humans ***(11)***. The reagents available to study inflammatory responses in rabbits are more limited, however. Dogs, pigs, and sheep permit physiological studies, but few species-specific reagents are available, and their large size requires large quantities of reagents ***(12–14)***. Primates respond similarly to humans, but are expensive and not widely available ***(15)***.

This chapter outlines methods to produce sepsis associated with either intrapulmonary or intraabdominal infections in rabbits. Methods are included for creating focal infections in the lungs or in the peritoneal cavity, for monitoring the physiological responses, and for assessing tissue-specific inflammatory and injury responses.

2. Materials

1. Animals: Specific pathogen-free female New Zealand white rabbits weighing 3.2–3.5 kg.
2. Equipment for monitoring physiological data using vascular catheters: Several different monitoring systems are available for the collection and analysis of physiological data. These systems typically include pressure transducers, a bridge amplifier and an analog to digital (A–D) converter. The bridge amplifier and the A–D converter amplify and convert the analog signal from the pressure transducer to a digital signal that can be processed by a computer. Software programs can then be used to display, manipulate, and store this data.
3. Anesthetics: Ketamine (Ketalar, 100 mg/mL; Parke-Davis, Morris Plains, NJ) and xylazine (Rompun, 100 mg/mL, Bayer Corp., Shawnee Mission, KS) are used for anesthesia.
4. Bacteria: The choice of bacteria for animal models of sepsis depends on the goals of the study. We have used *Escherichia coli*, because it is one of the most common clinical isolates in humans with sepsis ***(5)***. The K-1 serotype is associated with invasive infection in humans and rabbits. Bacteria are thawed from a frozen stock and inoculated into 50 mL Lennox-B broth, then grown overnight to stationary phase at 37°C with continuous stirring. After overnight incubation, the

bacteria are recovered by centrifugation, washed once in phosphate buffered 0.9% NaCl (PBS), and resuspended in sterile water to the desired concentration. Virulence can be maintained by routinely passing the bacteria in the same animal species used for the studies. For the passage of bacteria, a peritoneal infection (**Subheading 3.4.**) is induced in a rabbit, and 24 h later the spleen is aseptically recovered. The spleen is homogenized in 0.9% NaCl, and then brought to 30% glycerol. The homogenate is stored in aliquots at –70°C. After each passage, the identity of the bacteria growing from the homogenate should be verified.

3. Methods

3.1. Surgical Instrumentation of Rabbits for Physiological Monitoring

The arterial and venous catheters are placed surgically under aseptic conditions. These catheters are used for hemodynamic monitoring, fluid and antibody treatments, and blood sampling.

3.1.1. Anesthesia

The surgical placement of vascular catheters and the instillation of bacteria, either intratracheally or intraperitoneally, require general anesthesia. Anesthesia is induced with a combination of ketamine and xylazine and maintained with additional doses of ketamine as needed. During the surgical procedure the rabbit breathes spontaneously through an endotracheal tube (3.0 mm ID, 4.2 mm OD, Kendall/Sheridan, Argyle, NY).

1. A 24-gauge catheter (Angiocath, Becton-Dickinson, Sandy, UT) is placed in the marginal ear vein for induction and maintenance of anesthesia.
2. To induce anesthesia a combination of ketamine (10 mg/kg, iv) and xylazine (3 mg/kg, iv) is required. This combination of ketamine and xylazine permits intubation.
3. For the maintenance of anesthesia during surgery or for the instillation of bacteria, additional boluses of ketamine (50 mg/mL, iv) are given as needed.

3.1.2. Catheterization of the Jugular Vein and Carotid Artery

1. The right ventral region of the rabbit's neck is shaved and scrubbed with a povidone-iodine solution.
2. The skin is incised and the jugular vein and carotid arteries are surgically exposed. The venous and arterial catheters are inserted into the right jugular vein and right carotid artery, respectively, then advanced 5 cm and sutured in place. Each catheter (0.8 mm OD, Intramedic PE 90 tubing, Becton-Dickinson, Sparks, MD) has a total length of 45 cm.
3. The incision is closed and a dressing is placed over the surgical site. The arterial and venous catheters are connected to pressure transducers for measurement of the central venous and arterial pressures.

Table 1
Trouble-shooting Specific Problems

Method	Problem	Possible Solutions
Clot Making	1. Fibrinogen will not go into solution	1. Do not heat sample. Stir for 30 min in 0.9% Saline.
	2. Fibrin clot is not homogeneous	2. Place syringe with fibrinogen/thrombin solution in a shaker for 30 min.
	3. Fibrinogen/thrombin solution does not clot	3. Freeze thrombin in aliquots and thaw each aliquot prior to use.
Animal Monitoring	1. Erratic pressure readings	1. Check to make sure that no air bubbles are in the pressure transducers. Add filters to remove electrical interference.
	2. No blood return from the arterial catheter	2. Make sure that the line is not kinked. If there is no kink then try repeated flushing of the catheter. If this does not work, instill a small amount of heparin.
Microbiology	1. Quantitative culture are negative	1. Make sure that the agar is not too hot.
	2. Cultures are all positive	2. Monitor for contamination of agar or dilutional water by always pouring an agar plate containing no bacteria.
Immunohistochemistry	1. No positive staining in tissue where antigen is expected.	1. Evaluate different fixatives to determine which results in the best preservation of the antigen of interest.

4. To maintain the patency of the venous catheter, 0.9% NaCl is infused (4 mL/h) through the venous catheter using an IV infusion pump (Ivion, EZ1, Englewood, CO).
5. The arterial catheter is pressurized at 300 mmHg for the entire study to ensure patency. The resistance of the pressure transducer will determine the amount of fluid administered through the arterial catheter.

6. For the first 1–2 h post-surgery, the rabbit is placed on a heated water blanket (Gaymar Industries, Solid State T pump, Orchard Park, NY) to prevent hypothermia, as anesthetics interfere with normal temperature regulation.
7. Immediately after the surgical placement of the catheters, the bacteria are inoculated into either the right lung or the peritoneal cavity.

3.3. Induction of E. Coli Pneumonia

Bacterial pneumonia is induced in rabbits with the intratracheal administration of a bacterial suspension. Bacteria are coadministered with 1% colloidal carbon (Pelikan, Hanover, Germany) to allow visualization of the instilled area at necropsy ***(16)***.

1. For the administration of the bacterial suspension the rabbit is placed in the right lateral recumbent position on a 20° incline (head up).
2. The bacterial suspension (0.5–1 mL) is administered blindly to the right lung with a 5 french catheter (AccuMark, Keene, NH) advanced through an endotracheal tube.
3. If anesthesia is required to complete the administration of bacteria, ketamine (50 mg/mL) is administered as needed.
4. Immediately after administering the bacteria, the rabbit is given 5–10 gentle breaths with an anesthetic bag and then kept in right lateral recumbent position for 15 min.

3.4. Induction of E. Coli Peritonitis

To induce peritoneal sepsis, a clot infected with bacteria is placed into the peritoneal cavity of rabbits. This method was first described in a canine model of septic shock ***(17)***.

1. The clot is made by adding 2 U/mL of human thrombin (Sigma Chemical Co., St. Louis, MO) to 1% bovine fibrinogen (fraction I, Type IV, Sigma) in sterile 0.9% NaCl.
2. The skin of the abdominal area is shaved and prepped with povidone-iodine solution and anesthetized with 1 mL of lidocaine.
3. A 1-cm skin incision is made on the midline approx 1 cm above the umbilicus.
4. A small incision is made through the linea alba into the peritoneum using a #15 scalpel blade.
5. A 16-gauge catheter is passed through the incision into the peritoneal cavity. The clot is instilled through the catheter, and approx 1 mL of material is retained in the syringe for quantitative culture.
6. The peritoneum and skin are closed with sutures and dressed with sterile gauze.

3.5. Fluid Administration

In both the pulmonary and peritoneal model of sepsis the decision to administer fluids is determined by specific physiological criteria. The amounts of

fluids required by an animal are recorded as cumulative fluid requirements over the course of the study.

1. The following criteria are used for the administration of fluids:
   a. Arterial pH <7.30;
   b. Arterial blood pressure (ABP) <75 mmHg, or a decrease in ABP from baseline >15%; and
   c. Central venous pressure (CVP) <–10 cm H_2O.
2. All animals receive 10 mL 0.9% NaCl immediately after surgery. During the observation period, additional boluses of 10 mL 0.9% NaCl are given when the above criteria are met. The frequency of fluid bolus administration depends on the ABP: every 15 min for ABP between 65 and 75; every 10 min for ABP between 55 and 64, and every 5 min for ABP below 55.
3. The following criteria are used to discontinue the administration of fluids
   1. Evidence of pulmonary edema, such as crackles on auscultation of the chest, or foamy respiratory secretions by mouth;
   2. A CVP greater than 10 cm H_2O.

3.6. Collection of Samples

At the end of each experiment rabbits are euthanized with an overdose of pentobarbital (120 mg/kg), and exsanguinated by intracardiac puncture. Successful intracardiac puncture should yield approx 100 mL blood/3 kg animal.

3.6.1. Blood Samples

Blood samples are used for many measurements, including arterial blood gases, leukocyte counts, cultures, and cytokine determinations. During the observation period, 1-mL blood samples can easily be obtained through the arterial catheter using a 3-way stopcock.

3.6.2. Bronchoalveolar Lavage

Bronchoalveolar lavage fluid can be used for the determination of leukocyte counts, quantitation of bacteria, and measurement of total proteins and cytokines.

1. Following euthanasia and exsanguination the trachea is isolated and cross-clamped and the trachea, lungs, and heart are removed en bloc.
2. The trachea and lungs are dissected free from the heart and surrounding tissue and a sterile plastic catheter is inserted into the middle portion of the trachea and secured with silk suture.
3. The right middle lung lobe is removed for culture. This lung lobe is weighed and homogenized and the homogenate is cultured using quantitative techniques.
4. The right main stem bronchus is cross-clamped with a hemostat and the left lung is lavaged with 5 separate 15-mL aliquots of 0.9% NaCl containing 0.6 m*M*

EDTA at 37°C. The left main stem bronchus is then cross-clamped and the right lung is lavaged using the same protocol.
5. A 1-mL aliquot of BAL fluid is removed from the respective lung lavages and processed for leukocyte counts and cultures.
6. The remaining BAL fluid is spun at 200*g* to pellet cells and aliquots of the cell free supernatant fluid are stored at –70°C.

3.6.3. Peritoneal Lavage

In peritoneal sepsis models, the peritoneum is lavaged and the lavage fluid is processed for cell counts quantitation of bacteria, and measurement of cytokines.

1. Following thoracotomy, the peritoneal cavity is lavaged once with 20 mL of 0.9% NaCl containing 0.6 m*M* EDTA.
2. The lavage fluid is processed in the same manner as the BAL fluid.
3. After the lavage procedure, the peritoneal cavity is inspected for remnants of the clot.

3.6.4. Tissue Processing

Tissue can be harvested and processed for histologic and ultrastructural changes, measurement of RNA and specific biochemical analyses.

1. Tissue fixation: The techniques used to evaluate tissue structure (e.g., light microscopy, confocal microscopy, or electron microscopy), will vary with the goals of the experiment. Proper tissue processing and fixation should focus on preserving tissue structure and tissue antigens.
   a. An important goal of fixation is to preserve the lungs in a normal state of inflation. This requires that the airways be inflated with either fixative or air at an inflation pressure of 15–20 cm H_2O. We use airway perfusion of fixative at 15 cm H_2O pressure to fix the lungs from rabbits with bacterial pneumonia. If the preservation of the airway and alveoli architecture in the natural-state is required, vascular perfusion of the fixative is recommended. Methods for the fixation of the lung using airway or vascular perfusion have been described in the literature ***(18)***.
   b. Tissue that is being used for immunohistochemistry should be processed within 24 h of fixation to minimize antigen degradation. We have found that tissue fixation in 4% paraformaldehyde results in better demonstration of the rabbit chemokine antigens in lung sections.
2. Processing tissue for total RNA: To process total RNA from lungs, cut the lung tissue into small cubes and immediately flash freeze in liquid nitrogen. This tissue can then be stored at –70°C until the RNA is extracted. When extracting total RNA from tissue, endogenous RNase must be inactivated in order to obtain intact RNA. A commonly used method for the preparation of total RNA from tissue uses guanidine isothiocyanate when homogenizing tissue and a CsCl gra-

dient to isolate the RNA ***(19)***. Recently, commercially available kits have become available for the purification of total RNA from tissue. These kits have eliminated the need for organic extractions and CsCl ultracentrifugation in the preparation of total RNA from tissue.

3. Homogenization of lung tissue for the measurement of cytokines, and myeloperoxidase: When cytokines and myeloperoxidase are isolated from lung tissue, care must be taken in order to maintain protein structure and function. Myeloperoxidase, an enzyme found in the azurophilic granules of neutrophils, is often used to estimate neutrophil numbers in lung tissue. Specific protocols for the homogenization of lung tissue for the measurement of cytokines and myeloperoxidase have been described in the literature ***(20)***.

3.7. Analysis of Samples

3.7.1. Bacterial Cultures

Quantitative analysis of biological fluids for bacteria is performed using quantitative and nonquantitative culture techniques.

1. Quantitative cultures are performed by diluting 100 μL of a sample (e.g., whole blood, or BAL fluid) in 900 μL of distilled water (dH_2O) and then 1:10 serial dilutions in dH_2O (3 or 4 dilutions are usually sufficient).
2. 100 μL of each dilution are incorporated into an agar pour plate and cultured overnight at 37°C. Quantitative colony counts are performed 12–18 h later using an optical colony counter.
3. To detect low levels of bacteria that might be missed with the quantitative technique, 5 μL of a biological fluid is inoculated into 5 mL of trypticase-soy broth and incubated overnight at 37°C. If bacterial growth is identified, the colonies are seeded onto a tryptic soy blood-MacConkey agar plate for further identification.

3.7.2. Cell Counts

Total and differential cell counts are performed on whole blood, BAL, and peritoneal fluid in order to characterize changes in leukocyte numbers in these biological fluids.

1. The cells in the biological fluids are stained with trypan blue for determination of viability and with crystal violet containing citric acid (0.5 gm crystal violet and 10.5 grams citric acid in 500 mL dH_2O) for cell counts.
2. The cells are counted on a hemacytometer.
3. Differential cell counts of the BAL and peritoneal fluids are performed on cytospin-prepared slides. Differential counts of leukocytes in peripheral blood are performed on blood smears. The slides are stained with Diff-Quik (American Scientific Products, McGaw Park, IL) and at least 200 cells are counted per sample to determine the percent of neutrophils, macrophages, lymphocytes, and other cells.

3.7.3. Protein Measurements

There are a number of biological markers measured in plasma or in bronchoalveolar lavage fluid that can be used for the detection of acute lung injury ***(21)***. To determine the extent of lung injury which occurs in septic rabbits we measured three proteins in BAL fluid collected from rabbits.

1. Total protein is measured using the bicinchoninic acid method (BCA assay: Pierce Co., Rockford, IL).
2. Albumin is measured by immunoassay using sheep antirabbit albumin (Bethyl A-120-104A) as capture antibody and biotinylated rat antirabbit albumin (Zymed 04-1640) as detecting antibody.
3. Ig*M* is measured by immunoassay using goat antirabbit IgM (Accurate Chemical and Scientific Corp. SBA 4020-01), as detecting antibody and biotinylated goat antirabbit Ig*M*, μ chain specific (Accurate SBA 4020-08) as capture antibody.

3.7.4. Bioassays and ELISA for Measurement of Inflammatory Mediators

Inflammatory mediators in biological fluids (e.g., BAL fluid and serum) can be measured with either bioassays or ELISA. A cytotoxicity bioassay using L929 cells has been used to measure TNF-α in biological fluids collected from rabbits ***(22)***. While the availability of rabbit specific reagents can be a limiting factor in the measurement of inflammatory mediators, rabbit specific immunoassays have been described for the chemokines IL-8, GRO, and MCP-1 ***(23–26)***.

4. Notes

1. Specific pathogen free rabbits should be used in these experiments to reduce the possibility of respiratory pathogens such as *Pasteurella multocida* influencing experimental results.
2. In order to maintain bacterial virulence it is important to pass the bacteria through the same species used in the studies. Aliquots of this passage are stored at –70°C in gasket cryovials and used only once to eliminate potential problems caused by repeated freeze thawing of bacteria.
3. To facilitate the placement of the endotracheal tube a small amount of lidocaine (0.1–0.2 mL) can be used to anesthetize the back of the oropharynx. This is accomplished by advancing the endotracheal tube to the back of the oropharynx and administering the lidocaine through the endotracheal tube.
4. It is important to protect the catheters that run from the rabbits to the pressure transducers to prevent them from being damaged by the rabbits.
5. While the intratracheal administration of bacteria with a 5 french feeding tube (**Subheading 3.3.**) results in pneumonia in the right lung, it is difficult to administer the bacterial suspension to the same portion of the right lung in every animal. To insure that the bacterial suspension is placed in the same lung lobe in all experimental animals, the bacterial suspension can be administered via the

biopsy channel of a pediatric bronchoscope ***(27)*** or the feeding tube can be passed with fluoroscopic guidance ***(16)***.

6. In order to be able to visualize the regions of the lungs instilled with bacteria we have added 1% colloidal carbon to the instillate. We have used Pelikan India Ink (Pelikan, Hanover, Germany) because it does not contain shellac, which is commonly found in other preparations of colloidal carbon.
7. When administering 10 mL fluid boluses it is recommended that the 0.9% NaCl be warmed to insure that the fluid therapy does not affect the animals temperature.
8. Biological fluids should be kept on ice and processed as soon as possible in order to decrease protein degradation.
9. Biological fluids should be stored as 200 μL aliquots in gasket cryovials to prevent freeze thawing of samples.

5. Summary

To study the mechanisms that link sepsis with ARDS, many animal models have been developed. In this chapter, a rabbit model of sepsis secondary to an intrapulmonary or intraabdominal infection has been described. One advantage of the rabbit model of sepsis is that this species produces the C-X-C chemokine, IL-8. In contrast, rodents, which are often used in studies of sepsis and ARDS, lack this important chemokine. A second advantage is the rabbit's size. This species is large enough so that the measurement of physiological parameters (e.g., mean arterial pressure, heart rate, etc.) is not difficult, but they are not so large that they require large quantities of precious reagents (e.g., recombinant proteins and MAbs). A disadvantage of the rabbit model is that there are fewer reagents (e.g., recombinant cytokines and MAbs) available for the study of inflammation in rabbits when compared to mice.

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References

1. Kollef, M. H. and Schuster, D. P. (1995) The acute respiratory distress syndrome. *N. Engl. J. Med.* **332,** 27–37.
2. Bachofen, A. and Weibel, E. R. (1982) Structural alterations of lung parenchyma in the adult respiratory distress syndrome. *Clin. Chest Med.* **3,** 35–56.
3. Tomashefski, J. F. (1990) Pulmonary pathology of the adult respiratory distress syndrome. *Clin. Chest Med.* **11,** 593–619.
4. Hudson, L. D., Milberg, J. A., Anardi, D., and Maunder, R. J. (1995) Clinical risks for development of the acute respiratory distress syndrome. *Am. J. Respir. Crit. Care Med.* **151,** 293–301.

5. Martin, T., Matute-Bello, G., Skerrett, S. J., and Frevert, C. W. (1999) Extrapulmonary sepsis and cytokines in lung injury and defense, in *Cytokines and Pulmonary Host Defense* (Nelson, S. and Martin, T. R., eds.), Marcel Dekker, New York. (In press).
6. Bernard, G. R., Artigas, A., Brigham, K. L., Carlet, J., Falke, K., Hudson, L. D., et al. (1994) Report of the American-European consensus conference on ARDS: definitions, mechanisms, relevant outcomes and clinical trial coordination. The Consensus Committee. *Am. J. Respir. Crit. Care Med.* **149,** 818–824.
7. Fowler, A. A., Hamman, R. F., Good, J. T., Benson, K. N., Baird, M., Eberle, D. J. et al. (1983) Adult Respiratory Distress Syndrome: risks with common predispositions. *Ann. Intern. Med.* **98,** 593–597.
8. Montgomery, A. B., Stager, M. A., Carrico, C. J., and Hudson, L. D. (1985) Causes of mortality in patients with the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* **132,** 485–489.
9. Fink, M. P. and Heard, S. O. (1990) Laboratory models of sepsis and septic shock. *J. Surg. Res.* **49,** 186–196.
10. Martin, M. A. and Silverman, H. J. (1992) Gram-negative sepsis and the adult respiratory distress syndrome. *J. Infect. Dis.* **14,** 1213–1228.
11. Fox-Dewhurst, R., Alberts, M., Kajikawa, O., Caldwell, E., Johnson, M. C. I., Skerrett, S. J., et al. (1997) Pulmonary and systemic inflammatory responses in rabbits with gram-negative pneumonia. *Am. J. Respir. Crit. Care Med.* **155,** 2030–2040.
12. Olson, N. C., Kruse Elliott, K. T., and Johnson, L. (1990) Effect of LY171883 on endotoxin-induced lung injury in pigs. *J. Appl. Physiol.* **69,** 1315–1322.
13. Gossage, J. R., Kuratomi, Y., Davidson, J. M., Lefferts, P. L., and Snapper, J. R. (1993) Neutrophil elastase inhibitors, SC-37698 and SC-39026, reduce endotoxin-induced lung dysfunction in awake sheep. *Am. Rev. Respir. Dis.* **147,** 1371–1379.
14. Eichacker, P. Q., Waisman, Y., Natanson, C., Farese, A., Hoffman, W. D., Banks, S. M., and MacVittie, T. J. (1994) Cardiopulmonary effects of granulocyte colony-stimulating factor in a canine model of bacterial sepsis. *J. Appl. Physiol.* **77,** 1–8.
15. Leturcq, D. J., Moriarty, A. M., Talbott, G., Winn, R. K., Martin, T. R., and Ulevitch, R. J. (1996) Antibodies against CD14 protect primates from endotoxin-induced shock. *J. Clin. Invest.* **98(7),** 1533–1538.
16. Doerschuk, C. M., Markos, J., Coxson, H-O., English, D., and Hogg, J-C. (1994) Quantitation of neutrophil migration in acute bacterial pneumonia in rabbits. *J. Appl. Physiol.* **77,** 2593–2599.
17. Quezado, Z. M., Natanson, C., Alling, D. W., Banks, S. M., Koev, C. A., Elin, R. J., Hosseini, J. M., Bacher, J. D., Danner, R. L., and Hoffman, W. D. (1993) A controlled trial of HA-1A in a canine model of gram-negative septic shock. *JAMA* **269,** 2221–2227.
18. Weibel, E. R. (1984) Morphometric and stereological methods in respiratory physiology including fixation techniques, in *Techniques in Respiratory Physiology*, Part 1. (Otis, A. B., ed.), Elsevier, New York, NY, P4/I, P401/1–P401/35.

19. Davis, L. G., Dibner, M. D., and Battey, J. F. (1986) Guanidine isothiocyanate preparation of total RNA, in *Basic Methods in Molecular Biology* (xx, xx, ed.), Elsevier, New York, NY, pp. 130–135.
20. Greenberger, M. J., Strieter, R. M., Kunkel, S. L., Danforth, J. M., Goodman, R. E., and Standiford, T. J. (1995) Neutralization of IL-10 increases survival in a murine model of Klebsiella pneumonia. *J. Immunol.* **155,** 722–729.
21. Pittet, J. F., Mackersie, R. C., Martin, T. R., and Matthay, M. A. (1997) Biological markers of acute lung injury: prognostic and pathogenetic significance. *Am. J. Respir. Crit. Care Med.* **155,** 1187–1205.
22. Martin, T. R., Ruzinski, J. T., Wilson, C. B., and Skerrett, S. J. (1995) Effects of endotoxin in the lungs of neonatal rats: age-dependent impairment of the inflammatory response. *J. Infect. Dis.* **171,** 334–344.
23. Johnson, M. C., Goodman, R. B., Kajikawa, O., Wong, V. A., Mongovin, S. M., and Martin, T. R. (1994) Cloning of two rabbit GRO homologues and their expression in rabbit alveolar macrophages. *Gene* **151,** 337–338.
24. Johnson, M. C., Kajikawa, O., Goodman, R. B., Wong, V. A., Mongovin, S. M., Wong, W. B., Fox-Dewhurst, R., and Martin, T. R. (1996) Molecular expression of the α-chemokine rabbit GRO in *Escherichia coli* and characterization of its production by lung cells *in vitro* and *in vivo*. *J. Biol. Chem.* **271,** 10,853–10,858.
25. Kajikawa, O., Goodman, R. B., Johnson, M. C., Konishi K., and Martin, T. R. (1996) Sensitive and specific immunoassays to detect rabbit IL-8 and MCP-1: cytokines that mediate leukocyte recruitment to the lungs. *J. Immunol. Methods* **197,** 19–29.
26. Kajikawa, O., Johnson, M. C., Goodman, R. B., Frevert, C. W., and Martin, T. R. (1997) A sensitive immunoassay to detect the alpha-chemokine GRO in rabbit blood and lung fluids. *J. Immunol. Methods* **205,** 135–143.
27. Jones, H. A., Clark, R. J., Rhodes, C. G., Schofield, J. B., Krausz, T., and Haslett, C. (1994) *In vivo* measurement of neutrophil activity in experimental lung inflammation. *Am. J. Respir. Crit. Care Med.* **149,** 1635–1639.