

Experimental Activation of Mast Cells and Their Pharmacological Modulation

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Abstract

The activation of mast cells is of pivotal importance in the pathogenesis of allergic conditions. Mast cell activation can provoke rapid increases in microvascular permeability, induce bronchoconstriction after blood flow, stimulate the recruitment and activation of other inflammatory cells, and has come to be associated with the processes of tissue remodeling and fibrosis. Such changes may be mediated by the release of a range of potent mediators of inflammation: preformed in secretory granules, or newly generated, or both.

There are major differences in the responsiveness to various stimuli and to pharmacological agents for mast cells from different body compartments. A method is presented here for the purification of mast cells from enzymatically dispersed human tissues. The methods described for the experimental activation of mast cells can be readily adapted to studies with cell lines or mast cells obtained through long-term culture.

Key Words: Mast cell; activation; modulation of mast cells.

1. Introduction

Mast cell activation is of pivotal importance in the pathogenesis of allergic conditions. Histological evidence for mast cell activation in bronchial tissues and the presence of increased concentrations of mast cell products, including histamine, tryptase, and prostaglandin D₂ (PGD₂), have been noted in bronchoalveolar lavage fluid and sputum from asthmatics (1,2). Even during relatively asymptomatic periods, there is an increased degree of mast cell activation in asthma, and levels may be dramatically increased following exposure to allergen (3,4). Similarly, allergen challenge of rhinitis results in increased release of mast cell products into nasal lavage fluid (5,6), and allergen challenge in the skin of sensitized subjects is associated with the secretion of these mediators in skin blister fluid (7).

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Mast cell activation can provoke rapid increases in microvascular permeability, induce bronchoconstriction after blood flow, stimulate the recruitment and activation of other inflammatory cells, and has come to be associated with the processes of tissue remodeling and fibrosis (reviewed in **Ref. [8]**). Such changes may be mediated by the release of a range of potent mediators of inflammation (**9,10**). These have been divided into two main classes: those which are preformed and released from secretory granules and those which are newly generated following mast cell activation. Prominent among the former group are histamine, proteases (including tryptase, chymase, and carboxypeptidase), and proteoglycans. Products of arachidonic acid metabolism and particularly PGD₂ and leukotriene C₄ (LTC₄) constitute the newly generated mediators. Mast cell cytokines may constitute a third category, in that they may be both preformed and newly synthesized. Those produced by human mast cells include IL-4, IL-5, IL-6, and TNF α .

Investigation of the stimuli, mechanisms of mast cell activation, and the means for pharmacological modulation have become important areas in the study of allergic disease. The activation of mast cells by allergen binding to specific IgE on high-affinity receptors (FC ϵ R1) is the best understood mechanism of mast cell activation. This process of 'immunological activation' may be mimicked experimentally using antiserum specific for IgE which can crosslink membrane-bound IgE. Calcium ionophores can also prove useful as experimental stimuli, and like anti-IgE, can induce the activation of all populations of mast cells. Other 'nonimmunological' stimuli ('secretagogues') for mast cell activation include substance P, vasoactive intestinal peptide (VIP), C5a and C3a, somatostatin, compound 48/80, morphine, pepstatin, eosinophil major basic protein (MBP), platelet activating factor, platelet factor 4, and very low density lipoproteins (reviewed in **Ref. [11,12]**); all these have differential effects on mast cells from different sources of tissue. The mast cell product tryptase can also stimulate the activation of human tonsil mast cells and perhaps act as an amplification signal with other stimuli (**13**). In contrast, substance P, a stimulus for human skin mast cell activation, fails to activate mast cells of tonsil, lung, or gut tissue (**12**).

Selecting an appropriate model system for the investigation of mast cell activation can present a challenge, given the extent of mast cell heterogeneity. There are major differences in responsiveness to various stimuli and to pharmacological agents for mast cells from different body compartments. Ideally, in seeking to investigate the behavior of mast cells in a particular site, the cells should be derived from tissues from that site. Various human tissues may be obtained from operating theatres (with appropriate local research ethics committee approval; *vide infra*), although it should be borne in mind that such tissues are generally from diseased subjects and may not be typical. It may be possible to obtain

'normal' tissue that has proven unsuitable for transplant surgery or nondiseased skin which is removed at circumcision, but otherwise tissues will be inflamed, damaged, or cancerous, or adjacent to such tissues. Cells and tissues from rodents have been widely employed in investigations of mast cell degranulation in vitro, with mast cells recovered from the peritoneum or from chopped lung tissue (14). However, important differences in mast cell behavior have emerged from studies with different species, and caution is required in extrapolating from findings with rodent mast cells to the situation in man.

Early investigations of the activation of tissue mast cell in vitro relied on mechanical processes of tissue dispersion. Better standardization of the technique may be achieved by incubating chopped tissues with proteases to generate a homogeneous cell suspension. A mixture of pronase, chymopapain, elastase, and collagenase has been employed (15), although a mixture of collagenase and hyaluronidase alone may be more effective (16). The proportion of mast cells within dispersed tissue preparations ranges from 0.5–10% depending on the tissues. Such preparations may be suitable for the study of mast cells where the product of activation is produced mainly by this cell type. Histamine has been widely employed as a marker for mast cell activation, and a method for its measurement is provided here. Mast cell tryptase or chymase could also be employed, or PGD₂. It is important to realize, however, that there may be differential release of mast cell products with different stimuli. Thus, whereas anti-IgE can induce the release of histamine, PGD₂, and LTC₄ from human skin mast cells, substance P can stimulate the secretion of histamine from these cells without apparent release of PGD₂ and LTC₄ (12).

It could be argued that the activation of mast cells in vivo occurs in the presence of other cell types, and that it may thus be appropriate to employ mixed cell preparations to study the process. In some circumstances, however, there are advantages to using a pure preparation of mast cells, e.g., where a mediator to be analyzed is also produced by other cell types. A method is presented here for the purification of mast cells from enzymatically dispersed human tissues that involves isolation by density sedimentation with Percoll, followed by a positive selection procedure with an anti-*c.kit* monoclonal antibody and Dynabeads (17). The long-term culture of mast cells from precursors in bone marrow, fetal liver, or blood does offer a means of obtaining preparations of mature mast cells of high purity, although the purchase of the growth factors may be expensive. Another approach could involve the use of a cell line with mast cell-like features. Some lines (e.g., HMC1) have proved of limited value on account of their immature phenotype. The stem cell factor-dependent LAD1 and LAD2 cell lines derived from a patient with mast cell sarcoma/leukemia have many of the characteristics of mature tissue mast cells (18) and can offer a useful model for studies with mast cells. The methods described here for the experimental activation of

mast cells can be readily adapted to studies with cell lines or mast cells obtained through long-term culture.

Various antiallergic drugs have been found to possess mast cell-stabilizing properties. These include ketotifen, sodium cromoglycate, salbutamol (**16**), some histamine H₁ receptor antagonists (**12**), and certain inhibitors of mast cell tryptase (**13,19**) and chymase (**20**). Major differences in responsiveness have been found for mast cells from different sources and from different species. The extent of mast cell heterogeneity calls for the use of several different mast cell models when investigating mast cell function and pharmacological control.

2. Materials

2.1. Equipment

1. Class I cabinet.
2. Forceps and scissors.
3. Centrifuge.
4. Shaking water bath.
5. Water bath.
6. Hot plate.
7. Spiramix.
8. Magnetic cell sorter (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany).
9. Histamine analyzer (The Reference Library, Copenhagen, Denmark) with glass fiber-coated plates (Lundbeck Diagnostics, Copenhagen, Denmark).
10. Plate washing apparatus.
11. Dry incubator.

2.2. Media and Buffers

1. Hank's balanced salt solution (HBSS):
 - 4 g NaCl.
 - 2.4 g Hepes.
 - 0.5 g glucose.
 - 500 mL distilled water.
 - 0.5 mL 0.2 g/mL KCl.
 - 0.5 mL 0.06 g/mL NaH₂PO₄.
2. Complete HBSS:
 - 100 mL HBSS.
 - 100 μ L 0.265 g/mL CaCl₂.
 - 100 μ L 0.102 g/mL MgCl₂.
3. HBSS culture medium:
 - 500 mL HBSS.
 - 2% FCS.
 - 1% (v/v) streptomycin and penicillin.
 - 1% fungizone.

4. Minimal essential medium (MEM) culture medium:
500 mL Eagle's MEM.
2% (v/v) fetal calf serum (FCS).
2% (v/v) streptomycin and penicillin.
1% (v/v) fungizone.
5. DMEM culture medium:
500 mL Dulbecco's modification of Eagle's medium.
2% (v/v) FCS.
1% (v/v) streptomycin and penicillin.
10% MEM nonessential amino acid solution.
1% (v/v) L-glutamine.
6. Phosphate-buffered saline (PBS) culture medium:
500 mL PBS.
2% (v/v) FCS.
1% (v/v) streptomycin and penicillin.
0.3 mg/mL deoxyribonuclease (DNase).
7. Digestion solution:
Tonsil:
10 mL MEM culture medium for every gram of tissue.
1.5 mg/mL collagenase.
0.75 mg/mL hyaluronidase.
10% FCS.

Lung*:
10 mL MEM culture medium for every gram of tissue.
2 mg/mL collagenase.
1 mg/mL hyaluronidase.
10% FCS.

Skin or synovium:
10 mL MEM culture medium for every gram of tissue.
2.5 mg/mL collagenase.
1.5 mg/mL hyaluronidase.
10% FCS.
25 mg/mL bovine serum albumin (BSA).
8. 0.5% (w/v) Alcian blue dye in saline.
9. 65% continuous Percoll gradient:
2.16 mL 10× conc. HBSS without Ca or Mg.
19.5 mL N Percoll (Sigma, St Louis, MO).
3.4 mL HBSS.
0.3 mg/mL DNase.

*For purification of lung mast cells (Methods 3.2), the digestion solution should contain 15 mg/mL DNase and 1 mM dithioerythritol.

10. Blocking buffer:
90 mL PBS.
10 mL FCS.
1 g BSA.
11. 0.4% (w/v) SDS (sodium dodecyl sulfate or lauryl sulfate) solution.
12. Coupling reagent:
0.5 mg/mL *O*-phthaldialdehyde.
5% (v/v) methanol.
2 mg/mL NaOH.
13. 58 mM HClO₄.

3. Methods

3.1. Regulations, Safety, and Ethical Considerations

3.1.1. Ethical Issues

Whereas in the past surgically removed tissues have been treated as ‘clinical waste’ from operating theaters and used with relatively few restrictions in research, such studies may now require the specific approval of the Local Research Ethics Committee. Issues of informed consent (donor or next of kin), record keeping, and tissue storage require consideration. In many cases an ‘unlinked, anonymized’ study design will be adequate, where findings are not linked to the identity of a given patient.

3.1.2. Health and Safety Precautions

Workers are advised to consult their Local Safety Office before proceeding. Processing of tissues should be carried out in a Class I safety cabinet. It is recommended that protective clothing for the chopping of tissues should include a Howie-style lab coat, latex or nitrile gloves which overlap the cuffs of the lab coat, a disposable plastic apron, surgical mask, and eye protection. Tissues should be transported in sealed containers. Human tissues should be disposed of safely (e.g., by incineration) according to local guidelines. All work surfaces and utensils should be cleaned thoroughly and disinfected after use. Vaccination of workers against Hepatitis B is advised.

3.2. Mast Cell Dispersion and Challenge (see Notes 1 and 2)

1. Collect fresh lung (at lung resection; >3 g), tonsil (at tonsillectomy; >3 g), skin (at circumcision; >1 g), and synovial tissue (at knee replacement operations; >3 g) in MEM.
2. Weigh tissue.
3. Remove blood vessels and keep only macroscopically normal tissue for experiment.
4. Cut tissue into a few pieces and squeeze blood out from lung tissue in a 100 mm × 20 mm Petri dish.

5. Wash twice with ~15 mL MEM culture medium.
6. Decant off the supernatant and add ~3 mL MEM culture medium to tissue and chop finely (approx. $0.5 \times 1 \times 2 \text{ mm}^3$) with a pair of scissors.
7. Collect tissue fragments in 50-mL centrifuge tubes (approx. 2.5 g tissue per tube).
8. Top up tubes to 50 mL with MEM culture medium and centrifuge at 500g for 6 min at 4°C.
9. Discharge supernatant and resuspend tissue pellet in about 10 mL MEM culture medium and top up tube with MEM culture medium and centrifuge as above.
10. Repeat step 9 twice more.
11. Resuspend tissue pellet in the digestion solution and incubate at 37°C in a shaking water bath for 65 min for tonsil, 70 min for lung, and 80 min for skin and synovial tissue.
12. Separate cells from undigested tissue debris by passing through two sets of nylon mesh filters of pore size 100 μM (nylon tea strainers are ideal).
13. Continue to digest the undigested tissue as before in order to obtain more cells if required.
14. Centrifuge the filtrate at 500g for 6 min at 4°C.
15. Aspirate supernatant, resuspend cells in about 10 mL MEM culture medium, top up tubes to 50 mL, and centrifuge as above.
16. Repeat the above washing procedure.
17. Resuspend the cells in about 10 mL MEM culture medium with 10% FCS and adjust the volume to 50 mL.
18. Leave the cells on a roller overnight at room temperature.
19. Centrifuge the tubes at 500g for 6 min at room temperature.
20. Resuspend the cells in 10 mL MEM culture medium.
21. To estimate the cell numbers, mix 10 μL of the cell suspension with 10 μL 0.5% Alcian blue dye in saline for 20 min at room temperature. Count total and positive stained cells with a hemocytometer. With an improved Neubauer hemocytometer, counting the middle large triple-ruled square, $\text{cells/mL} = \text{cell numbers counted} \times 10^4 \times 2$ (dilution factor). Add 40 mL more MEM culture medium to the cell suspension and leave on roller at room temperature until use.
22. Add 25 μL secretagogue and 25 μL pharmacological agonist (or complete HBSS) in 4-mL polypropylene tubes on ice (for spontaneous and total histamine release, add 50 μL complete HBSS into each tube).
23. Centrifuge the cell suspension at 500g for 6 min at 25°C.
24. Aspirate the supernatant, resuspend cells in about 10 mL HBSS + 2% FCS, top up tubes to 50 mL, and centrifuge as above.
25. Repeat above.
26. Aspirate the supernatant, resuspend cells in about 10 mL HBSS, top up tubes to 50 mL, and centrifuge at 500g for 6 min at 25°C.
27. Resuspend cells in complete HBSS to the desired volume (so that 100 μL of cell suspension contains 2500–5000 mast cells).
28. Prewarm mast cell suspension and tubes in 37°C water bath for approx. 5 min.
29. Add 100 μL cell suspension into each tube and mix them carefully.

30. Incubate tubes for 15 min.
31. Place tubes on ice and immediately add 150 μ L cold HBSS in each tube.
32. Boil the tubes for total histamine release for 6 min, then put them on ice.
33. Centrifuge all tubes at 900g for 10 min at 4°C.
34. Transfer the supernatants into capped 1.5-mL tubes and store at -20°C until assaying for histamine.

3.3. Purification of Lung Mast Cells (see Note 3)

The whole procedure should be carried out under sterile conditions:

1. Collect fresh lung tissue (>15 g) and maintain in HBSS.
2. Weigh tissue in tared 50-mL container.
3. Remove blood vessels and bronchi from tissue.
4. Cut lung tissue into a few pieces and gently squeeze blood out from tissue in a 100 mm $\times$ 20 mm Petri dish.
5. Wash tissue twice with about 15 mL of HBSS culture medium.
6. Add approx. 3 mL HBSS culture medium to lung tissue and chop finely (to pieces approx. 0.5 $\times$ 1 $\times$ 2 mm³) with a pair of scissors.
7. Collect tissue fragments in 50-mL centrifuge tubes (approx. 2.5 g tissue per tube).
8. Top up tubes to 50 mL with HBSS culture medium and centrifuge at 500g for 6 min at 4°C.
9. Discharge supernatant and resuspend tissue pellet in about 15 mL HBSS culture medium and top up tube to 50 mL with HBSS culture medium and centrifuge as above.
10. Wash tissue twice with 50 mL HBSS culture medium.
11. Resuspend tissue pellet in 50 mL DMEM culture medium and leave on roller overnight at 4°C.
12. Centrifuge at 500g for 6 min at 4°C.
13. Resuspend tissue pellet in lung digestion solution (containing 15 mg/mL DNase and 1 mM dithioerythritol). Incubate at 37°C in a shaking water bath for 90 min.
14. Separate cells from undigested tissue debris by passing through two sets of nylon mesh filter of pore size 100 μ M (nylon tea strainers are ideal).
15. Redigest the undigested tissue as before in order to obtain more cells if required.
16. Centrifuge the filtrate at 500g for 6 min at 4°C.
17. Resuspend cells in about 10 mL MEM culture medium, and divide into two 50 mL centrifuge tubes. Top up tubes to 50 mL and centrifuge as above.
18. Combine all the cells in a single 50-mL tube and wash twice with 50 mL MEM culture medium.
19. Resuspend the cells gently in a small volume of DMEM and adjust to 5 mL.
20. Mix 5 mL cell suspension with 25 mL Percoll and centrifuge at 1400g for 20 min at 4°C (if cells from more than 15 g tissue used, then increase the quantity of Percoll).
21. Remove supernatant and transfer into a number of 50-mL tubes -5 mL per tube.
22. Aspirate red blood cells and wash tube with small volume of PBS culture medium in order to recover more mast cells.

23. Add 30 mL PBS culture medium to each tube, mix gently, and centrifuge at 500g for 10 min at 4°C.
24. Combine all cells and wash twice more with 30 mL PBS culture medium.
25. Resuspend cells in 1 mL PBS culture medium and mix 10 μ L of it with 0.5% Alcian blue solution for 20 min at room temperature.
26. Count total and positive stained cells with a hemocytometer (as in **Section 3.1.**).
27. Add 2 mL blocking buffer to 1 mL of cell suspension, then add 30 μ L 5 mg/mL IgG (final concentration 50 μ g/mL).
28. Incubate the mixture for 30 min on ice on a roller.
29. Top up the mixture with 30–40 mL PBS culture medium and centrifuge at 500g for 10 min at 4°C.
30. Aspirate the supernatant and resuspend cells in 600 μ L blocking buffer.
31. Add 15 μ L of 5 μ g/mL monoclonal antibody YB5B8 (specific for *c.kit*; Pharmingen, San Diego, CA) to the cells dispersed from 15 g tissue and mix carefully, ensuring the cells do not clump.
32. Incubate the mixture for 30 min on ice on a roller.
33. Disperse the clumps with a pipet and incubate for a further 30 min.
34. Meanwhile:
 - a. Add 30 μ L of Dynabeads to 20 mL PBS culture medium in a universal container and expose the container to a magnetic field for 3 min.
 - b. Aspirate the supernatant and resuspend the Dynabeads in 20 mL PBS culture medium and expose to a magnetic field for a further 3 min. Repeat twice more.
 - c. Resuspend Dynabeads in 500 μ L blocking buffer.
35. Centrifuge the cells at 1000 rpm, 4°C for 10 min in a microcentrifuge.
36. Resuspend cells with Dynabeads solution at a ratio of 5–8 beads per mast cell, mix well with a pipet, and incubate the mixture for 90 min on ice on a roller (disperse cells every 30 min).
37. Dilute the mixture to 20 mL with PBS culture medium and expose to magnetic field for 3 min.
38. Aspirate the supernatant and repeat step 37 above thrice more.
39. Resuspend the cells in DMEM for culture or HBSS culture medium for challenge.
40. Stain cells with 0.5% Alcian blue solution in saline for 20 min at room temperature. Count the total and positive stained cells with a hemocytometer.

3.4. Histamine Measurement (see Note 4)

1. Add 50 μ L sample, 50 ng/mL histamine standard, or HBSS to each well of 96-well glass fiber coated plate.
2. Incubate in dry incubator for 60 min at 37°C.
3. Wash plate with distilled water using plate washing apparatus for 2 min.
4. Add 150 μ L 0.4% SDS solution to each well.
5. Incubate in dry incubator for 30 min at 37°C.
6. Wash plate as above.
7. Add 75 μ L coupling reagent to each well.

8. Incubate in dark for 10 min at room temperature.
9. Add 75 μ L 58 mM HClO_4 to each well to stop reaction.
10. Read plate on a histamine analyzer.

Histamine release should be expressed as a percentage of total cellular histamine levels and corrected for the spontaneous release measured in tubes in which cells had been incubated with the HBSS diluent alone (i.e., percentage net histamine release = [(histamine release with stimulus – spontaneous histamine release)/total histamine content] $\times$ 100.

4. Notes

1. The procedure for tissue chopping and mast cell dispersion and challenge is here described as a continuous procedure that may be carried out in a single working day. However, chopped tissues (step 7) may be maintained overnight at 4°C on a mechanical roller, and the cell dispersion and challenge can take place the following day. This has advantages to the worker when large numbers of experiments are to be performed or when tissues are received late in the day. Incubating the tissue overnight may also lead to lower spontaneous histamine release in subsequent mast cell challenge experiments.
2. The number of experiments that may be carried out at one time can depend on the skill and the experience of the worker. The novice may prefer to restrict themselves to some 20–30 tubes, but several hundred are possible (in which case it is essential that tubes for estimation of spontaneous or total histamine are included at regular points throughout and the potential for a drift in values assessed).
3. The yield and purity of the mast cells are generally difficult to predict at the outset of such experiments, and there may be a trade-off between the two. Whereas it is possible to obtain mast cells more than 95% pure, the proportion of contaminating cells may be much greater in some cases. Differences can reflect differences between lung tissues and the skill of the worker.
4. Other methods for the measurement of histamine may be considered, such as specific enzyme immunoassay (Beckman Coulter, Fullerton, CA) which is more sensitive but expensive if many experiments are planned. The measurement of other specific mast cell products (e.g., tryptase, chymase, PGD_2) may also be considered, and there are commercially available assays. For certain products of mast cell activation produced by other cell types also (e.g., LTC_4 , cytokines), it may be necessary to restrict studies to purified cells. Cytokine release from activated mast cells may be increased many hours following addition of the stimulus, and so longer incubation periods should be employed when cytokine release is to be measured (ideally several time-points).

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