

Generation, Isolation, and Maintenance of Rodent Mast Cells and Mast Cell Lines

UNIT 3.23

This unit presents protocols for the culture of mast cells from a variety of rodent sources. Protocols for the isolation and culturing of mast cells from other sources (e.g., human surgical specimens) are described in UNIT 7.25. These mast cell models can be employed for investigating ligand-dependent mast cell mediator release, chemotaxis, and adhesion. They are also suitable for investigating mechanisms of signal transduction associated with these responses. Protocols for examining mediator release are also described in UNIT 7.26, and these can be utilized or adapted for use with the techniques for obtaining rodent mast cells described in this unit.

The protocols described in this unit will allow the reader to obtain relatively pure populations of mast cells from the peritoneal cavity of rat and mouse (Basic Protocol 1) and to generate mouse bone-marrow-derived mast cells (BMMCs; Basic Protocol 2). The latter approach is applicable to obtaining BMMCs from wild-type, knockout, and transgenic mice, irrespective of the strain. Basic Protocol 2 also provides steps for the characterization of mast cells by staining and flow cytometry. In addition, information is included on routine culturing of the rat basophilic leukemia (RBL-2H3) mast cell line (Support Protocol 1) and the mouse MC/9 mast cell line (Support Protocol 2). Details will also be provided for the characterization of mast cells by staining and flow cytometry.

ISOLATION AND CULTURE OF PERITONEAL MAST CELLS

BASIC PROTOCOL 1

Rodent peritoneal mast cells (PMCs) are of the connective tissue phenotype (see Commentary) that exhibits a mature morphology and high granularity (Metcalf et al., 1997). They express the high-affinity receptor for IgE (FcεRI) which can be occupied by IgE following in vivo sensitization and restimulated ex vivo with antigen; the ex vivo restimulation can also occur following in vitro sensitization. After activation, PMCs release a variety of pro-inflammatory mediators including histamine, serotonin, eicosanoids, chemokines, and cytokines (Metcalf et al., 1997). The percentage of mast cells within the peritoneal cavity ranges between 5% and 10%, with the remainder of cells being primarily of the macrophage/monocyte lineage, as determined by metachromatic staining with toluidine blue and flow cytometric analysis. The following protocol describes the isolation of rat mast cells by ex vivo lavage of the peritoneal cavity and subsequent purification using a Percoll gradient technique. The peritoneal lavage can be conducted on a benchtop, since the cavity is not open and the cells will be maintained in a sterile environment. The subsequent purification of mast cells is conducted in a laminar flow hood. The total cell yield ranges from 10 to 20 × 10⁶ mixed peritoneal cells and from 0.4 to 2 × 10⁶ mast cells per rat, depending on the size and age of the rat. The number of mast cells routinely obtained is always greater in ovalbumin (OVA)-sensitized rats. The total cell yield from mice ranges from 1 to 3 × 10⁶ mixed peritoneal cells, 3 to 6 × 10⁵ of which will be mast cells (Coleman et al., 1993; Eastmond et al., 1997).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations for the care and use of laboratory animals.

Materials

- 10× and 1× Hanks' balanced salt solution (HBSS) without Ca²⁺ or Mg²⁺ (e.g., Invitrogen) with phenol red
- Rat (150 to 200 g, 3 to 6 months old) *or* mouse (20 g, 2 to 6 months old)

In Vitro Assays
for Mouse
Lymphocyte
Function

70% ethanol
 0.8% (w/v) M NH₄Cl in double-distilled H₂O
 70% isotonic Percoll solution (see recipe)
 PMC culture medium (see recipe)
 0.4% (w/v) trypan blue in PBS (see APPENDIX 2A for PBS)
 0.05% (w/v) toluidine blue in PBS (see APPENDIX 2A for PBS)
 10-ml syringes (for mice) or 30-ml syringes (for rats)
 23-G needles (for mice) or 19-G needles (for rats)
 Dissecting instruments: scissors and forceps
 15- and 50-ml conical polypropylene centrifuge tubes (Falcon)
 Centrifuge
 3-ml pipet (Corning)
 Additional equipment and reagents for euthanasia of rodents (UNIT 1.8), counting cells using a hemacytometer (APPENDIX 3A), trypan blue exclusion test of cell viability (APPENDIX 3B), and toluidine blue staining of mast cells (UNIT 7.25)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All procedures are performed in a laminar flow hood. All reagents are sterilized by filtration and all surgical equipment sterilized by immersion in 70% ethanol prior to use.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

Prepare syringes loaded with 1 × HBSS

- 1a. *For rats:* Fill two 30-ml syringes with 1 × Ca²⁺- and Mg²⁺-free HBSS and attach a 19-G needle to each. Put aside until required.
- 1b. *For mice:* Fill two 10-ml syringes with 1 × Ca²⁺- and Mg²⁺-free HBSS and attach a 23-G needle to each. Put aside until required.

Perform peritoneal lavage

2. Anesthetize rat/mouse using a rising concentration of carbon dioxide and then euthanize by cervical dislocation (UNIT 1.8). Place the rat/mouse on its back, bathe the ventral area in 70% ethanol, and remove the skin from the abdominal area using forceps and scissors.
3. Bathe the exposed abdomen with 70% ethanol, then pinch and lift the abdominal wall away from the internal organs using forceps. Be sure to keep the peritoneal cavity intact.

This will create an empty space within the peritoneal cavity where the needle can be placed for the injection of HBSS.

4. Take one of the HBSS-preloaded syringes, turn the needle beveled side up and pass it through the still intact peritoneal cavity directly under the forceps where the space was created in the previous step. Gently push on the plunger of the syringe until all of the HBSS has been expelled.

Be careful not to puncture any internal organs with the needle or over-inflate the cavity with HBSS, since this can cause internal bleeding. The peritoneum should be left intact to ensure sterility.

5. Vigorously agitate the abdominal area for 60 sec by gently squeezing the top of the cavity (below the diaphragm) and the bottom of the cavity (above the hips) with both hands to ensure that the HBSS distributes throughout the peritoneum.

6. Place the animal on its side to allow the fluid to settle.
7. Reinsert the needle, beveled side down, at the bottom center of the abdomen and gently recover the fluid by withdrawing the plunger of the syringe.

Take care not to catch internal organs or fat when recovering HBSS from the peritoneal cavity, as this will contaminate the cell suspension. The recovered liquid should be yellow in color, indicating the acidity of the cavity, and cloudy, indicating the presence of cells. If there are problems in recovering HBSS, turn the animal on its other side and repeat this step.

8. Repeat the lavage procedure (steps 3 to 7) with the other preloaded syringe.
9. Under laminar flow hood/sterile conditions, pool the peritoneal cell suspensions in sterile 50-ml conical polypropylene centrifuge tubes. Centrifuge 5 min at $400 \times g$, room temperature.

Isolate mast cells

10. Remove the supernatant and resuspend the cells in the small amount of remaining solution. If the cell pellet is red, remove the red blood cells by one of the following techniques. If necessary, repeat until cell pellet is white.
 - a. *For rat:* Add 18 ml double-distilled water to promote osmotic lysis, immediately followed by 2 ml of $10\times$ HBSS to return the solution to iso-osmolarity. Centrifuge cell suspension 5 min at $400 \times g$, room temperature.
 - b. *For mouse:* Add 1 ml of 0.8% NH_4Cl solution, let stand 5 min, then add 14 ml of $1\times$ HBSS. Centrifuge cell suspension 5 min at $400 \times g$, room temperature.

Osmotic lysis will damage mouse peritoneal cells.

11. Remove the supernatant from the resulting white cell pellet, add 5 ml of $1\times$ HBSS to the pellet, and wash by centrifuging 5 min at $400 \times g$, room temperature, and removing the supernatant. Resuspend the pellet in 8 ml of 70% isotonic Percoll solution.
12. Gently overlay the 70% isotonic Percoll cell solution with 2 ml PMC medium at room temperature and centrifuge 15 min at $580 \times g$, room temperature.

A 70% Percoll solution has been determined to yield the greatest number of mast cells with the highest purity.
13. Using a 3-ml pipet, carefully remove the top (macrophage/monocyte) layer and discard.
14. Using another 3-ml pipet, remove the remaining Percoll solution without disturbing the mast cell pellet at the bottom of the tube.
15. Resuspend the mast cell pellet with 100 μl PMC medium prewarmed to 37°C , and transfer to a clean 15-ml conical tube containing 10 ml prewarmed PMC medium.

Count viable cells and mast cells

16. Centrifuge cells 5 min at $400 \times g$, room temperature. Remove supernatant and resuspend the mast cells in 1 ml of 37°C PMC medium.
17. Add 10 μl cell suspension to 10 μl of 0.4% trypan blue solution, then count the total numbers of live and dead cells, respectively, using a hemacytometer (APPENDIX 3A).

Dead cells will take up the dye and stain blue while live cells will exclude the dye and be clear. Cells are routinely $>99\%$ viable. APPENDIX 3B provides additional detail on the trypan blue exclusion test for cell viability.

18. Add 10 μ l cell suspension to 10 μ l of 0.05% toluidine blue solution and incubate at 37°C for 10 min. Count the total number of nucleated cells and mast cells using a hemacytometer (*APPENDIX 3A*).

Mast cells will stain dark blue while non-mast cells will be clear. Mast cell purity should be >98%. Also see UNIT 7.25.

19. Finally, adjust cells to $0.5\text{--}1 \times 10^6$ total nucleated cells per ml, place 1-ml aliquots in 15-ml conical centrifuge tubes, and incubate no longer than 48 hr in a humidified 37°C, 5% CO₂ incubator.

GENERATION AND CULTURING OF MOUSE BONE MARROW-DERIVED MAST CELLS AND CHARACTERIZATION BY FLOW CYTOMETRY

Mouse bone marrow-derived mast cells (BMMC) belong to the mucosal phenotype (see Commentary). They can be sensitized by IgE and activated, following antigen stimulation, in a manner similar to the PMCs (see Basic Protocol 1) BMMCs can be generated from progenitor cells found in the bone marrow by culturing them in IL-3-rich medium. It takes 4 weeks to generate BMMCs and the yield is about 100×10^6 cells from one mouse (two femurs). Using mice older than 6 months may decrease the yield.

This protocol describes how to obtain mouse bone marrow cells, culture them into mature mast cells, and determine their purity by flow cytometry analysis after staining for Fc ϵ RI and CD117 (Kit). The functionality can be determined by using a release assay which measures β -hexosaminidase, histamine (see *UNIT 7.26*) or serotonin (see *UNIT 7.26*).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations for the care and use of laboratory animals.

Materials

Mouse (2 to 6 months old)
70% ethanol
BMMC–MC/9 culture medium (see recipe), prewarmed to 37°C
0.05% (w/v) toluidine blue in PBS (see *APPENDIX 2A* for PBS)
Phosphate-buffered saline (PBS; *APPENDIX 2A*) containing 0.1% (w/v) BSA, 4°C
Anti-Fc ϵ RI-FITC (eBioscience, cat no. 11-5898) and isotype control, hamster
IgG-FITC (eBioscience, cat. no. 11-4444) *or* anti-CD117-PE (BD Pharmingen, cat. no. 555714) and isotype control, mouse IgG₁-PE (BD Pharmingen, cat. no. 555749)

Dissecting instruments: scissors and forceps
6-well tissue culture plates (Costar)
30-ml syringes
25-G needles
125-cm² tissue culture flask (Sarstedt, Nunc)
50-ml conical polypropylene centrifuge tubes (Falcon)
Flow cytometer tubes: 5-ml polystyrene round-bottom tubes
Flow cytometer (Chapter 5)

Additional reagents and equipment for euthanasia (*UNIT 1.8*), counting cells using a hemacytometer (*APPENDIX 3A*), toluidine blue staining of mast cells (*UNIT 7.25*), and flow cytometry (Chapter 5)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All procedures, including bone removal and femur lavage, are performed in a laminar flow hood. All reagents are sterilized by filtration through an 0.22- μ m filter and all surgical equipment are sterilized by immersion in 70% ethanol prior to use.

NOTE: All incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

Harvest bone marrow

Additional detail on harvesting mouse bone marrow is provided in *UNIT 22B.1*.

1. Disinfect scissors and forceps with 70% ethanol. Sacrifice a mouse by CO₂ asphyxiation (*UNIT 1.8*) and bathe it in 70% ethanol. Remove femurs using the scissors and forceps and place the femurs in 10 ml of 37°C BMMC–MC/9 culture medium.

Clean the bone as much as possible by cutting off the attached muscle with scissors. This makes it easier to flush the bone.

2. Transfer the femurs and the 10 ml BMMC–MC/9 culture medium into a well of a 6-well plate. If femurs still contain a lot of attached muscles, remove them. Cut the ends of the femurs so that the bones can be flushed. Add 5 ml BMMC–MC/9 culture medium to another well.

3. Fill a syringe with 37°C BMMC–MC/9 culture medium and attach a 25-G needle to the syringe. Using the syringe and needle, flush the bone marrow out of the interior of each femur and into the well containing the 5 ml of medium.

The bone becomes clear when the red marrow is removed.

4. Transfer the medium containing the bone marrow cells to a 125-cm² culture flask. Rinse the well with 5 ml of 37°C BMMC–MC/9 culture medium and add this to the same culture flask. Add a further 40 ml of 37°C BMMC–MC/9 culture medium to the flask and culture cells in a humidified 37°C, 5% CO₂ incubator for 3 to 4 days.

Culture BMMC

5. Change the medium twice a week (Monday and Friday) as follows. Transfer the cell suspension to a 50-ml conical polypropylene centrifuge tube and centrifuge 5 min at 450 $\times$ g, room temperature. Remove the supernatant, resuspend the cell pellet in 50 ml fresh BMMC–MC/9 culture medium and transfer to a new 125-cm² culture flask.

Replace the culture flask every time the medium is changed, to remove contaminating adherent cells. This is especially important at the beginning of culture when many adherent cells are present.

6. After 2 weeks, when the bone marrow cells start to proliferate, adjust the cell density to 1×10^6 cells/ml.

Determine mast cell purity

At week 4, the cell culture consists of mature BMMCs, and the cells can be utilized for experiments.

- 7a. *To evaluate purity by toluidine blue staining:* Add 10 μ l of 4-week cell suspension to 10 μ l of 0.05% toluidine blue solution. Incubate at 37°C for 10 min, then count the total number of nucleated cells and mast cells using a hemacytometer (*APPENDIX 3A*).

See UNIT 7.25 for additional information on toluidine blue staining.

The BMMCs should not be used past 7 weeks in culture, when the reactivity begins to decrease.

To evaluate purity by surface staining of FcεRI and Kit using flow cytometry

- 7b. Centrifuge a sample containing 100,000 cells for 5 min at $450 \times g$, room temperature.
- 8b. Resuspend cells in PBS containing 0.1% BSA, at 4°C, to 1×10^6 cells/ml, then add 100 µl of the cell suspension per flow cytometer tube.
- 9b. Add anti-FcεRI-FITC (or its isotype control, hamster IgG-FITC) or anti-CD117-PE (or its isotype control; mouse IgG1-PE) and incubate 30 min at 4°C in the dark.

Approximately 1 µl of each antibody stock is added, but antibody titration is recommended before the start of the experiment.
- 10b. Wash off excess antibodies by adding 1 ml PBS containing 0.1% BSA, at 4°C, to each tube, centrifuging 5 min at $450 \times g$, 4°C, and removing the supernatant. Resuspend cell pellet in 100 µl PBS containing 0.1% bovine serum albumin (BSA) and analyze on a flow cytometer (Chapter 5).

SUPPORT PROTOCOL 1

SPLITTING AND SUBCULTURING OF RBL-2H3 MAST CELL LINE

RBL-2H3 is an adherent mast cell line that expresses FcεRI and which releases mediators such as histamine following FcεRI aggregation (Barsumian et al., 1981). Stable cell lines have also been produced by transfecting the parental RBL-2H3 cells with both the human FcεRI (Gilfillan et al., 1992) and m1 muscarinic receptor (Jones et al., 1991).

RBL-2H3 is cultured as a monolayer. When the cell layer is approaching confluency, the culture has to be replated at a lower density (i.e., split, or subcultured). Generally, this is done twice a week as described below.

Materials

Near-confluent culture of RBL-2H3 cells (ATCC no. CRL-2256; see Support Protocol 3 for instructions on defrosting cell sample and establishing culture)
RBL-2H3 culture medium (see recipe)
0.25% trypsin-EDTA (e.g., Invitrogen)
RBL-2H3 culture medium (see recipe) containing 10% (v/v) DMSO
Liquid nitrogen
Inverted microscope (optional)
50-ml conical polypropylene centrifuge tubes (Falcon)
Tabletop centrifuge
Culture flasks
Cryotubes (e.g., Nunc)
Liquid nitrogen freezer (Dewar flask and canes to accommodate cryotubes)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

1. Remove the culture medium from the RBL-2H3 cells and briefly rinse the cell monolayer with prewarmed (37°C) 0.25% trypsin/EDTA. Discard this rinse solution to remove traces of fetal bovine serum (FBS) left over from the culture medium. Add sufficient (0.5 to 4 ml) trypsin/EDTA (depending on the size of culture flask) to just cover the cells and place the flask in an incubator at 37°C until cells detach.

2. To determine if cells have detached, examine the flask by holding up to a light or using an inverted microscope.

The plastic becomes clear and the medium becomes turbid once cells detach. Sharply hitting the side of the flask can help in detaching the cells.

3. Add 40 ml RBL-2H3 culture medium to each flask, transfer contents to a 50-ml conical polypropylene centrifuge tube, and centrifuge for 5 min at $450 \times g$, room temperature. Resuspend the cell pellet in 40 ml RBL-2H3 culture medium, centrifuge again as before, and remove the supernatant.
4. Resuspend pellet in 10 ml RBL-2H3 culture medium. Dilute cells 1:10 to 1:20 in a total volume of 20 ml medium for a 75 cm^2 flask or 40 ml for a 175 cm^2 flask, and add the cell suspensions to new culture flasks.

If nonadherent cells appear before cells reach confluency, remove medium (and the floating cells) and replace with fresh medium.

Cell lines should not be subcultured for longer than 3 months, to avoid cellular changes. Cell aliquots should therefore be cryopreserved (see next step).

5. **Cryopreserve cells:** Prepare a cell pellet as described in steps 1 to 3, above, resuspend in RBL-2H3 culture medium containing 10% (v/v) DMSO at a concentration such that the suspension can be distributed at $5\text{--}10 \times 10^6$ cells per cryotube, and freeze in liquid nitrogen for long-term storage.

Different laboratories have used up to 50% FBS in the freezing medium.

SPLITTING AND SUBCULTURING OF MC/9 MAST CELL LINE

The mouse MC/9 mast cell line is derived from mouse fetal liver. It is a suspension cell line, and growth is dependent upon IL-3, which is a component of the BMMC–MC/9 culture medium (see Reagents and Solutions). MC/9 cells express Fc ϵ RI and can be activated by cross-linking of these receptors, resulting in release of mediators including histamine (Nabel et al., 1981; Ishizuka et al., 1996).

Materials list

MC/9 cells (ATCC no. CRL-8306; see Support Protocol 3 for instructions on defrosting cell sample and establishing culture)
BMMC–MC/9 culture medium (see recipe)
BMMC–MC/9 culture medium (see recipe) containing 10% (v/v) DMSO
Liquid nitrogen
50-ml conical polypropylene centrifuge tubes (Falcon)
Tabletop centrifuge
Culture flasks
Cryotubes (e.g., Nunc)
Liquid nitrogen freezer (Dewar flask and canes to accommodate cryotubes)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All incubations are performed in a humidified 37°C , 5% CO_2 incubator unless otherwise specified.

1. Collect the cell suspension in a 50-ml conical polypropylene centrifuge tube and centrifuge cells for 5 min at $450 \times g$, room temperature. Remove supernatant.
2. Resuspend the cell pellet in fresh BMMC–MC/9 culture medium so that the cell concentration is $2\text{--}5 \times 10^5$ cells/ml.

SUPPORT PROTOCOL 2

**In Vitro Assays
for Mouse
Lymphocyte
Function**

3.23.7

**SUPPORT
PROTOCOL 3**

3. Change medium twice weekly as described in steps 1 and 2. Do not allow the cell density to exceed 2×10^6 cells/ml.

Cell lines should not be subcultured for longer than 3 months, to avoid cellular changes. Cell aliquots should therefore be cryopreserved (see next step).

4. **Cryopreserve cells:** Prepare a cell pellet as described in steps 1, above, resuspend in BMMC–MC/9 culture medium containing 10% (v/v) DMSO at a concentration such that the suspension can be distributed at $5\text{--}10 \times 10^6$ cells per cryotube, and freeze in liquid nitrogen for long-term storage.

STARTUP OF CULTURES FROM FROZEN ALIQUOTS

Materials

Frozen aliquot of cells (Support Protocol 1 or 2)
Cell culture medium
50-ml conical polypropylene centrifuge tubes (Falcon)
Tabletop centrifuge
Additional reagents and equipment for counting cells using a hemacytometer
(APPENDIX 3A)

1. Thaw the cell sample and immediately transfer the suspension to a 50-ml conical tube containing 30 ml cell culture medium.
2. Centrifuge cells 5 min at $450 \times g$, room temperature. Remove supernatant and resuspend pellet in 10 ml culture medium.
3. Count cells (APPENDIX 3A) and adjust cell concentration to the optimal concentration for the given cell line (see Basic Protocol 1 or 2).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see APPENDIX 5.

BMMC–MC/9 culture medium

825 ml RPMI 1640 medium (e.g., Invitrogen)
100 ml fetal bovine serum (FBS; 10% v/v final)
20 ml 200 mM L-glutamine (e.g., Invitrogen; 4 mM final)
10 ml 10,000 U/ml penicillin/10,000 µg/ml streptomycin (e.g., Invitrogen; 100 U/ml penicillin/100 µg/ml streptomycin final)
25 ml 1 M HEPES (25 mM final)
10 ml 100 mM sodium pyruvate (1 mM final)
10 ml 100× nonessential amino acids (e.g., Invitrogen; 1× final)
50 µM (final concentration) 2-mercaptoethanol (2-ME)
30 ng/ml (final concentration) murine IL-3 (Peprotech)
Mix all above components together, filter sterilize using a 0.2-µm filter and store up to 2 weeks at 4°C. Before use, prewarm to 37°C.

Other culture conditions have been reported for MC/9, such as:

1. DMEM (with 4.5 g/liter glucose, 1.5 g/liter sodium bicarbonate) supplemented with 10% FBS, 2 mM L-glutamine, 50 µM 2-mercaptoethanol and 10% Rat T-STIM (BD Biosciences).

2. RPMI 1640 supplemented with 20% (v/v) 1× WEHI-3B conditioned medium (see UNIT 22F.5 for preparation), 15% FBS, nonessential amino acids, 50 U/ml penicillin/50 µg/ml streptomycin, and 50 µM 2-mercaptoethanol.

Mast Cells

3.23.8

Isotonic Percoll solution, 70%

7 ml Percoll (Amersham Biosciences), sterile
1 ml 10× Hanks' Balanced Salt Solution (HBSS; e.g., Invitrogen), sterile
0.1 ml FBS (10% v/v final), sterile
1.9 ml double-distilled H₂O, sterile

Under sterile conditions, add all reagents to a 15-ml conical tube, vortex, and use immediately. Always make up fresh on day of PMC isolation and discard unused solution.

The recipe may be scaled up accordingly for larger volumes.

PMC culture medium

462.5 ml Dulbecco's modified Eagle's medium (DMEM; e.g., Invitrogen)
25 ml FBS (5% v/v final)
5 ml 200 mM L-glutamine (e.g., Invitrogen; 2 mM final)
2.5 ml 10 mg/ml gentamicin (50 µg/ml final)
5 ml 1 M HEPES (20 mM final, to give a pH of 7.4)
Mix all above components together, filter sterilize using a 0.2-µm filter and store up to 2 weeks at 4°C. Prior to use, prewarm to 37°C.

RBL-2H3 culture medium

435 ml Iscove's Modified Dulbecco's medium (IMDM; e.g., Invitrogen)
50 ml FBS (10% final)
5 ml 200 mM L-glutamine (e.g., Invitrogen; 2 mM final)
10 ml 10,000 U/ml penicillin/10,000 µg/ml streptomycin (e.g., Invitrogen; 100 U/ml penicillin/100 µg/ml streptomycin final)
Mix all above components together, filter sterilize using a 0.2-µm filter, and store up to 2 weeks at 4°C. Before use, prewarm to 37°C.

RBL-2H3 cells have also been reported to have been cultured with 15% FBS and in DMEM.

COMMENTARY

Background Information

Mast cells are key activators of the clinical manifestations associated with the allergic response. These cells develop from CD34/CD117 (Kit)/CD113-positive cells which originate in the bone marrow. The subsequent growth, development, and survival of mast cells in the peripheral tissues require stem cell factor (SCF)-induced ligation of its receptor, Kit (Metcalf et al., 1997). Two major mast cell phenotypes are distinguished in the mouse and human, as characterized by the presence of key granule components and their localization to specific target tissues. Rodent mast cells have been divided into the following classifications—connective tissue (serosal) mast cells (particularly in skin and peritoneal cavity) and mucosal mast cells (particularly in the intestinal lamina propria). It has been reported that the rodent mast cells express different proteases; connective tissue mast cells contain a high level of MCP4, 5 and 6, whereas mucosal mast cells have MCP 1

and 2 (Stevens et al., 1994). Human mast cells are normally categorized (by their protease content) into a population that contains tryptase and chymase (MC_{TC}) and one that primarily contains tryptase (MC_T). Regarding tissue localization, the human MC_{TC} is most similar to the rodent connective tissue mast cells, whereas the human MC_T correspond closely to the rodent mucosal mast cells (Metcalf et al., 1997).

The major stimulus for activation of mast cells in vivo is antigen-mediated FcεRI aggregation. However, other endogenous agents, including ligands for Toll-like receptors (TLRs), chemokines, adenosine, complement component C3a, nerve growth factor, SCF, and corticotrophin-releasing hormone, by binding to their specific receptors, can potentiate antigen-mediated mast cell activation, or by themselves induce mast cell activation (Gilfillan and Tkaczyk, 2006). The extent to which these receptors contribute to mast cell-dependent disease states in vivo is

nevertheless currently unknown. Mediators released from activated mast cells are either preformed or synthesized de novo upon receptor ligation/aggregation. Preformed mediators, which are stored in the large cytosolic metachromatic-staining granules, include histamine, serotonin (5-hydroxytryptamine; primarily rodent), β -hexosaminidase, tryptase, chymase, and other proteolytic enzymes. The contents of histamine, serotonin, and β -hexosaminidase in the supernatants of activated mast cells have all been used as indices of mast cell degranulation.

Arachidonic acid metabolites (eicosanoids), primarily leukotriene (LT) C_4 and prostaglandin (PG) D_2 , are de novo synthesized and released from activated mast cells as a consequence of phospholipase A_2 -catalyzed hydrolysis of phosphatidylcholine and phosphatidylethanolamine. These responses have been monitored by labeling cellular phospholipids to equilibrium with radiolabeled (primarily ^{14}C -labeled) arachidonic acid, then monitoring the counts released into the supernatants after challenge. Alternatively, the release of LTC $_4$ and PGD $_2$ has been monitored by measuring these compounds in the supernatants of challenged cells via ELISA. The abovementioned responses are generally measured within 1 to 30 min following challenge, as maximal degranulation and eicosanoid production are observed within this time frame. Finally, the generation of chemokines and cytokines in activated mast cells can be monitored at both the mRNA and protein levels. Messenger RNA levels are generally measured 2 to 4 hr after challenge. The most comprehensive, but not necessarily the easiest and quickest approach to monitoring the mRNA levels for multiple cytokines, is by the RNase protection assay (RPA; UNIT 10.29) using commercially available kits. Changes in the mRNA levels can be qualitatively monitored by RT-PCR or semiquantitatively measured by real time-PCR. The release of cytokine and chemokine protein into the supernatants of activated mast cells can also be monitored by ELISA.

The pivotal role of mast cells in allergic reactions associated with asthma and other disorders has led to development of in vitro systems to characterize receptor-mediated mast cell activation. Early studies of mast cell activation relied primarily on the isolation of mast cells from the peritoneal cavity of rats. Although sufficient numbers of cells are obtained to allow the investigation of mediator release in response to particular stimuli, the

numbers are not optimal for signaling studies. This issue led to the use of cell lines for these studies. The most widely used mast cell line is RBL-2H3. This name (which stands for Rat Basophilic Leukemia) is somewhat of a misnomer since the cells are more mast cell-like than basophil-like. The RBL-2H3 cell line proved to be invaluable for the delineation of the structure and immediate signaling events associated with the Fc ϵ RI (Kinet et al., 1987; Beaven and Metzger, 1993). In addition, RBL-2H3 cells could be readily transfected, which allowed the generation of cell lines expressing the human Fc ϵ RI and other native and chimeric receptors (Gilfillan et al., 1992; Repetto et al., 1996). This not only allowed for the delineation of the role for specific molecules and the contribution of specific receptor domains to the initiation of signaling, but also provided the pharmaceutical industry with a suitable model for high-throughput screening of potential inhibitors of mast cell activation. The major drawback to this cell line is the fact that it possesses an activating mutation in Kit (codon 815) equivalent to that associated with mastocytosis (codon 816) in humans. Thus, signaling studies may be complicated by a background activation of signals, such as phosphoinositide 3-kinase (PI3K), mediated by the activated Kit. A mouse mast cell line, MC/9, has also been used for signaling studies, but not to the same extent as the RBL-2H3 cells. As with the RBL-2H3 cells, MC/9 is transformed cell line; therefore, the inherent interpretational issues that exist with studies conducted in RBL-2H3 cells also pertain to this model.

The most widely used model of mouse mast cell function is BMMC. With the advent of gene-knockout mice and transgenic mice expressing mutant forms of specific signaling molecules, there has been an upswing in the popularity of this model. Unlike human mast cells, which are cultured in the presence of SCF and IL-6, the generation and culture of mouse BMMCs in vitro can be achieved by culturing cells in medium containing IL-3 alone, or in medium supplemented with IL-3 and SCF. It has been reported that these different culture conditions skew the phenotype from largely mucosal-like (IL-3) to more serosal-like (SCF). There are many advantages to use of BMMCs. For example, the cells are easy to grow and maintain in culture; from one femur it is possible to obtain $\sim 50 \times 10^6$ cells; therefore, cell number is not a factor limiting experimental design. Furthermore, the cost of culture on a dollars per million cells basis is

significantly lower than that required to grow human cells. Another benefit is that, unless genetically modified, there should be no underlying polymorphisms/mutations that may complicate conclusions made from observed results. Techniques are available for transfecting these cells, and, finally, data obtained from mouse BMMCs can be corroborated in vivo by examining such mast-cell-dependent responses such as PCA (passive cutaneous anaphylaxis) and PSA (passive systemic anaphylaxis) reactions in genetically modified mice similar to those from which the bone marrow was obtained. The primary drawback of growing mouse BMMCs is the 4 weeks required to obtain mature mast cells and the fact that there is an increased potential for bacterial and fungal infection during the processing of the bone marrow and extended culture. A further consideration is species-species variation between the mouse and human. Regardless, a survey of the literature reveals that the use of mouse BMMC, and, to a lesser extent, human mast cells, for the study of mast cell biology has largely overtaken studies utilizing rat and mouse cell lines.

The major current application of studies utilizing mouse BMMC, as alluded to above, has been for the delineation of critical signaling events involved in receptor-mediated mast cell activation. As reviewed by Gilfillan and Tkaczyk (2006), there is an increasing supply of mice available with genes for specific signaling molecules knocked out; thus, the manifestations of these deletions in mast cell function can be readily monitored by growing mast cells from the bone marrow of these animals. Due to concerns related to compensation mechanism, an alternative approach has been to generate transgenic mice expressing null mutations of the protein of interest (Ali et al., 2004). With recent advances in siRNA/shRNA technology for gene knock-down, where knockout mice are not available, it may be cheaper and certainly quicker to obtain a definitive answer about the role of newly identified gene products in mast cell function using either mouse BMMCs or human mast cells, instead of generating knockout mice. A further application of siRNA/shRNA is in cases where a particular knockout may be developmentally lethal. Regardless, the use of BMMCs obtained from knockout mice has resulted in an exponential increase in understanding with regard to the complexities of mast cell activation over the last 10 years (Gilfillan and Tkaczyk, 2006).

Critical Parameters and Troubleshooting

The most commonly encountered problem with the protocols described in this unit is bacterial, mycoplasmal, yeast, and fungal contamination. Fortunately, visible evidence of these issues (apart from mycoplasma contamination) is readily apparent. Commercially available kits are useful to monitor for mycoplasmal contamination (*APPENDIX 3E*), which is usually suspected when cells are growing more slowly than expected. The simplest solution to these problems is continual surveillance and sound aseptic technique. The addition of antibiotics to the culture medium should minimize problems with bacterial contamination; however, the use of fungicides should be avoided. It goes without saying that any tissue culture flask or dish showing signs of contamination should be immediately disinfected and discarded, and steps should be taken to wipe the surface in contact with the contaminated container with 70% ethanol or similar sterilizing agent. In addition to mycoplasma, a further reason for slow BMMC growth could be problems with the FBS or IL-3. It is advisable to test specific lots of FBS for optimal growth prior to the purchase of larger quantities. The IL-3 should be aliquoted and kept frozen (preferably -80°C) until required. This is generally added to the culture medium fresh, but once made up it can be kept at 4°C for up to a week prior to use. RBL-2H3 cells may spontaneously become unresponsive to antigen on prolonged passage. Should this happen, after all other possibilities have been ruled out, a fresh batch should be started. Once sufficient expansion has taken place, and assuming that normal degranulation is observed in response to antigen, further aliquots of cells (at $5\text{--}10 \times 10^6/\text{ml}$) in freezing medium should be frozen down in liquid nitrogen.

A high background (spontaneous) secretion and/or a high background in signaling studies in either RBL-2H3 cells or mouse BMMCs, assuming all reagents have been prepared correctly, is likely a reflection of the DNP-IgE, which is used for sensitization, becoming aggregated. To minimize this problem, the authors of this unit avoid multiple freeze-thaw cycles and spin the IgE at $14,000 \times g$ at 4°C for 45 min prior to storage at 4°C . Normally the IgE is stable for many months at this temperature.

Starving BMMCs by culturing them for 4 to 12 hr in medium without IL-3 is recommended when studying signal transduction, to avoid

Table 3.23.1 Anticipated Amounts of Released Mediators in Rodent Mast Cell Activation Assays

Assay	Cells per well (100–200 μ l)	Anticipated release
β -hexosaminidase	50,000–250,000	20%–50%
Histamine release	5000–10,000	30%–50%
LTC ₄	10,000	400–600 pg/well
Cytokine release	200,000	IL-6: 2000–4000 pg/ml IL-13: 2000–3000 pg/ml TNF α : 500–1000 pg/ml
Chemokine release	200,000	MCP1: 250–500 pg/ml

potential interpretational issues related to the IL-3 background signals.

Anticipated Results

Regarding cell yields, the number of PMCs from one rat would be $0.4\text{--}2 \times 10^6$; the corresponding number from one mouse would be $3\text{--}6 \times 10^5$. The number of BMMCs after 4 weeks culture would be $\sim 100 \times 10^6$ cells.

Regarding cell yields from RBL-2H3 and MC/9, $\sim 30\times$ expansion over 4 days may be expected.

The number of cells that are required for different activation assays (during an IgE/antigen response) and the anticipated amounts of released mediators are given in Table 3.23.1. The number of cells per well is based on cells cultured under the normal conditions described in the protocols in this unit. IgE (anti-DNP) and DNP-HSA (100 ng/ml of each) are used for activation of the cells. For measuring mediators, the supernatants have to be diluted between 1:2 and 1:10 for cytokines and chemokines, and 1:100 for LTC₄.

Time Considerations

The time required for the isolation of PMCs is 4 hr and these are used within 4 days. The time required to generate BMMCs is 4 weeks. For optimal results, the cells should be used before they turn 7 weeks, since at this age they begin to lose reactivity.

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