

Mast Cell Histamine and Cytokine Assays

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Summary

Mast cells are crucial to the development of chronic allergic inflammation and are likely to play a critical role in host defense. In this chapter methodology for histamine and cytokine assays is provided. Crosslinkage of IgE receptor I (FcεRI) on cord blood-derived mast cells by myeloma IgE and anti-human IgE is used to induce histamine release. Histamine levels were measured in the culture supernatants using an enzyme-linked immunosorbent assay. A human mast cell line (HMC-1), derived from a patient with mast cell leukemia, was activated with interleukin (IL)-1β to study cytokine production and gene expression. Cytokine gene expression was evaluated by reverse transcriptase polymerase chain reaction and cytokine production was assayed in culture supernatants using an enzyme-linked immunosorbent assay kit.

Key Words: Mast cells; cord blood-derived mast cells; IgE; IgE receptor; histamine; inflammation; enzyme-linked immunosorbent assay; reverse transcription polymerase chain reaction (RT-PCR); cytokines; RNA, IL-8.

1. Introduction

Mast cells are critical to the development of chronic allergic inflammation, as seen in allergic asthma (1,2). Crosslinking of cell surface IgE bound to the high-affinity IgE-receptor I, FcεRI, leads to the rapid release of inflammatory mediators, including histamine, proteases, chemotaxins, and arachidonic acid metabolites, such as prostaglandins and leukotrienes (3). Studies from several laboratories, including our own, have demonstrated the capacity of these cells to express certain cytokines regulating the inflammatory response (4–7). Through their production of cytokines, chemokines, and inflammatory mediators, mast cells play an important role in various inflammatory and neoplastic states (8).

In this chapter, crosslinkage of FcεRI by myeloma IgE and anti-human IgE are used to induce histamine release from human cord blood-derived mast cells

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(CBDMCs [7]). Histamine levels will be measured in the culture supernatants using the enzyme-linked immunosorbent assay (ELISA). The procedure of growing human CBDMCs is detailed in Chapters 8 and 9. In this chapter, CBDMCs and human mast cell line (HMC-1) derived from a patient with mast cell leukemia (9) were activated with the cytokine interleukin (IL)-1 β to induce IL-8 production and gene expression. IL-8 gene expression was evaluated by reverse transcriptase polymerase chain reaction (RT-PCR) whereas cytokine levels in culture supernatants were assayed by ELISA.

2. Materials

2.1. Histamine Release From Human CBDMCs by Crosslinkage of Fc ϵ RI With Myeloma IgE and Anti-Human IgE

2.1.1. Crosslinkage of Fc ϵ RI With Myeloma IgE and Anti-Human IgE

1. Human CBDMC media: DMEMF12 (Gibco Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (Atlanta Biologicals, Atlanta, GA); 5×10^{-5} M 2-mercaptoethanol (Fisher, Pittsburgh, PA); 0.5 mL of insulin–transferin–sodium selenite solution (Sigma-Aldrich, St. Louis, MO); 25 mM HEPES (Gibco, Carlsbad, CA); 300 nM PGE₂ (Cayman, Ann Arbor, MI); 100 ng/mL recombinant human IL-6 (kindly provide by Amgen, Thousand Oaks, CA); and 80 ng/mL stem cell factor (kindly provided by Amgen [7]).
2. 75-cm² flasks culture flasks, vented cap (Corning, Corning, NY).
3. Myeloma IgE (Calbiochem, San Diego, CA).
4. Anti-human IgE (Mouse IgM monoclonal antibody, Hybridoma Reagent Laboratory, Baltimore, MD).
5. Phosphate-buffered saline (PBS), pH 7.2.
6. Tyrode's solution: 124 mM NaCl, 4 mM KCl, 0.64 mM NaH₂PO₄, 1 mM CaCl₂, 0.6 mM MgCl₂, 10 mM HEPES buffer, and 0.03 % human serum albumin, pH 7.4.
7. Phorbol myristate acetate (PMA; 1 μ g/mL stock solution, Sigma).
8. Centrifuge tubes (15 mL).
9. 12-well culture plate (Falcon).

2.1.2. Measurement of Histamine by ELISA

1. Immunotech Histamine Enzyme Immunoassay Kit (Beckman Coulter, Fullerton, CA), which includes a 96-well microtiter plate with lid (coated with monoclonal anti-histamine antibody), standards (six vials containing histamine ranging from 0 to 100 nM), control (one vial of histamine of known concentration), acylation reagent (one vial), dimethyl sulfoxide (one 3-mL vial to dissolve acylation reagent), acylation buffer (one 5-mL vial), histamine-release buffer (one vial to be reconstituted with 25 mL of distilled water), histamine–alkalin phosphatase conjugate (one vial to be reconstituted with conjugate diluent H), conjugate diluent H (one 25-mL vial), wash solution 20X (one 50-mL vial to be diluted to 1 L with distilled water), substrate buffer (one 30-mL vial), substrate (two 15-mg tablets of Para-nitrophenylphosphate, pNPP, to be dissolved in 30 mL of substrate buffer), and stop solution (one 6-mL vial of 1 N NaOH solution).

2. Plastic tube (15 mL and 50 mL).
3. Micropipets (20 μ L, 200 μ L, and 1000 μ L).
4. Vortex-type mixer.
5. Microtiter plate shaker (350 rpm).
6. Microtiter plate washer (Bio-Tek, Winooski, VT).
7. Microtiter plate reader (405–414 nm, Dynatech MR5000, Chantilly, VA).
8. BioLinx 2.22 software (Dynex Technology, Chantilly, VA).

2.2. Growth of HMC-1 Cells

1. HMC-1 culture media: RPMI 1640 media supplemented with 2×10^{-5} M 2-mercaptoethanol (Sigma), 10 mM HEPES, gentamycin 50 μ g/mL, 5 mg/mL insulin transferrin (Sigma), 2 mM L glutamine, and 5% heat-inactivated fetal bovine serum.
2. 75-cm² culture flasks, vented cap (Corning, Corning, NY).
3. Sterile serological pipet tips and pipets.
4. Incubator (37°C and 5% CO₂).
5. Cell culture hood.

2.3. Cytokine Production and Gene Expression in IL-1 β -Activated HMC-1 Cells

2.3.1. Activation of Mast Cells With IL-1 β

1. IL-1 β (Sigma).
2. Micropipets and tips.
3. Cell culture media (*see Subheading 2.2.*).

2.3.2. RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction

1. Micropipets (p200 and p1000) and tips.
2. Centrifuge tubes (1.5 mL).
3. Microcentrifuge and large centrifuge.
4. Ice and bucket.
5. RNA-Bee.
6. Chloroform (at 0°C).
7. Phenol-cholorform (*see Note 1*).
8. Tris-HCl (sterile, 0.1 and 0.5 M, pH 8.0).
9. *n*-propanol.
10. 75% ethanol + 0.1% DEPC.
11. 0.1% diethyl pyrocarbonate (DEPC) H₂O.
12. Spectrophotometer.
13. RT-PCR Kit (GeneAmp RNA PCR Core Kit, Applied Biosystems, Roche, Branchburg, NJ).

2.3.3. Cytokine Production in Culture Supernatants Using ELISA

1. Cytokine ELISA Kit (R&D Systems, Minneapolis, MN).
2. Same as **steps 2–8** in **Subheading 2.1.2.**

3. Methods

The methods described in **Subheadings 3.1.–3.3.** outline: (1) histamine release from human CBDMC by crosslinkage of FcεRI with myeloma IgE and anti-human IgE; (2) growth of HMC-1 cells; and (3) cytokine gene and protein expression in IL-1β-activated HMC-1 cells. For the method of growth of CBDMC, please *see* Chapters 8 and 9.

3.1. Histamine Release From Human CBDMCs by Crosslinkage of FcεRI With Myeloma IgE and Anti-Human IgE

3.1.1. Crosslinkage of FcεRI With Myeloma IgE and Anti-Human IgE

1. Human CBDMCs are developed from human cord blood and maintained in CBDMC media for approx 8–16 wk or until mature (7).
2. Pipet 10 mL of CBDMC (5×10^5 cells/mL in CBDMC media) into each of the 75-cm² flasks. Treat CBDMC with 0 or 2 μg/mL final concentration of myeloma IgE by adding 0 (media control) or 20 μL of myeloma IgE (1 mg/mL) to the flask, and incubate at 37°C in a 5% CO₂ incubator overnight.
3. Pipet myeloma IgE-treated CBDMC into 15-mL centrifuge tubes (*see Note 2*) and centrifuge the tubes at 250g for 10 min at room temperature. Resuspend the cell pellet in 11 mL of PBS and do a cell count. Centrifuge the cell suspension and make up cell suspension at 1×10^6 cells/mL in Tyrode's solution.
4. Distribute 1.5 mL of CBDMCs into each well of 12-well culture plate in triplicate. Add anti-human IgE antibody to the wells at a final concentration of 0 (media control) or 3 μg/mL (i.e., adding 0 or 2.25 μL of 2 mg/mL anti-IgE, respectively). For positive controls, CBDMCs are treated with PMA at 50 ng/mL final concentration (adding 75 μL of 1 μg/mL PMA). The culture is further incubated for 30 min.
5. The cell cultures are harvested, centrifuged at 250g for 10 min, and cell-free supernatants are collected and kept at –80°C for histamine assay. Cell pellet is suspended in 1 mL of distilled water, frozen and thawed twice, and centrifuged at 250g for 10 min. Cell lysates are collected and kept at –80°C for histamine assay (for determination of cell residual histamine content).
6. The experimental results are expressed as the percentage of histamine release relative to the total cellular histamine content. Histamine release (%) = $[(E - S) / (E + L)] \times 100$, where E (experimental release) is the histamine in the experimental sample, S (spontaneous release) is the histamine in the sample of cells in buffer, and L (cell lysate) is the histamine in cell lysate sample.

3.1.2. Measurement of Histamine by ELISA

1. Equilibrate reagents of the kit to room temperature before use.
2. Reconstitute: (1) acylation reagent in the required volume of dimethyl sulfoxide just before use; and (2) conjugate solution with diluent H. Also, dissolve one tablet of substrate in 15 mL of substrate buffer at least 30 min before use.

3. Perform acylation of standards, control, and samples: add 25 μL of acylation solution, 100 μL of standards, control and samples, and 25 μL of acylation buffer to clean plastic tubes, and vortex immediately.
4. Add 50 μL of acylated standard, control, or samples and 200 μL of enzymatic conjugate into antibody-coated wells (*see Note 3*), and incubate for 2 h at 2–8°C with constant shaking on a microtiter plate shaker.
5. Wash the microtiter plate wells three times with the wash solution using a microtiter plate washer. Alternatively, the washing can be carried out manually as following: 1) turn microtiter plate upside-down and shake vigorously over the sink, 2) fill the wells with the wash solution using a squeeze bottle, 3) repeat **steps 1** and **2** twice for a total of three washes, and 4) firmly tap the upside-down microtiter plate on clean absorbent paper.
6. Add 200 μL of substrate to all wells, cover plate with lid, and incubate for 30 min at 18–25°C with constant shaking.
7. Stop the reaction by adding 50 μL of stop solution to all wells.
8. Read the optical density (OD) of the wells at 405–414 nm using a microtiter plate reader, zero with the substrate blank (*see Note 4*).
9. Results are obtained by interpolation from standard curve, which is generating from BioLinX 2.22 software (*see Note 5*).

3.2. Growth of HMC-1 Cells

HMC-1 cells, established from a patient with mast cell leukemia, were graciously provided by Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN [9]).

1. Cells (1 mL) taken out of the freezer are thawed, diluted in media (10 mL), and centrifuged to remove freezing media.
2. Reconstitute cell pellet to $1\text{--}2 \times 10^5$ cells/mL. Mast cells should be grown in vented cap culture flasks in a proliferating state at an optimal concentration of 2×10^5 cells/mL to 4×10^5 cells/mL (6,10,11). Overcrowding of cells will decrease their viability.
3. Subculture cells every 3–4 d to a 1:15 dilution with fresh new media. Culture flasks should be laid on their bottom (not standing up) to maximize media surface area.

3.3. Cytokine Production and Gene Expression in IL-1 β-Activated HMC-1 Cells

3.3.1. Activation of Mast Cells With IL-1 β

1. Make a cell suspension at a concentration of 1×10^6 cells/mL.
2. One milliliter of HMC-1 is seeded in tissue culture dishes or 12- or 24-well tissue plates, depending on the experimental design. Cells in media alone serve as negative controls. A combination of PMA (50 ng/mL) and ionomycin (5 μM) is used as a positive control.
3. For IL-1β activation, add varying concentrations of IL-1β (1, 10, and 100 ng/mL) to wells containing mast cells, and swirl in a figure eight motion to evenly distribute the stimulant (6,10,11).

- For cytokine production, mast cell cultures are incubated at 37°C for 0, 24, and/or 48 h (as required), harvested in 1.5-mL centrifuge tubes, and centrifuged at 250g for 10 min to remove cell debris.
- Cell-free supernatants are transferred to new tubes and stored at -70°C until ready for cytokine assay by ELISA (see **Note 6**).
- For ribonucleic acid (RNA) extraction, activation is conducted for 3 h in tissue culture plates (see **Note 7**). The cells (a total of 2–4 million cells will give sufficient RNA) are then harvested and subjected to RNA preparation and analysis. RNA can be extracted at 0, 3, 6, 9, 12, or 24 h as needed (depending on experimental condition).

3.3.2. RNA Extraction

- Centrifuge cells in 15-mL centrifuge tubes, discard supernatants (or save as the case may be), break pellet by flicking the bottom of the tube.
- Add 1 mL of RNAzol to broken cell pellet and pipet up and down until a watery consistency is obtained. Transfer to 1.5-mL centrifuge tubes.
- Add 100 μ L of cold chloroform and shake for 1 min. Do not vortex.
- Centrifuge for 15 min at 12,000g at 4°C.
- Transfer top aqueous layer to a new 1.5-mL centrifuge tube (see **Note 8**).
- Add approx 800 μ L of phenol–chloroform and vortex.
- Centrifuge for 5 min at 12,000g at 4°C.
- Repeat **steps 5–7**.
- Transfer top aqueous layer to a new 1.5-mL centrifuge tube and add approx 800 μ L of cold chloroform to the sample. Vortex the tube.
- Centrifuge for 5 min at 12,000g at 4°C.
- Transfer top layer to a new 1.5-mL centrifuge tube and add 800 μ L of cold n-propanol. RNA should start to precipitate. Vortex. Store at -20°C overnight.
- The next day, centrifuge samples for 30 min at 12,000g at 4°C.
- Discard supernatant and add 1 mL of 75% ethanol + 0.1% DEPC.
- Vortex gently until RNA pellet rises and falls intact.
- Centrifuge for 8 min at 10,000g at 4°C.
- Discard supernatant and using a p200 micropipet remove any trace of liquid from RNA pellet.
- Allow RNA pellet to air dry for 10 min at room temperature (see **Note 9**).
- Add 20 μ L of 0.1% DEPC H₂O to dissolve RNA. Read optical density on a spectrophotometer (see **Note 10**).
- Apply 1000 ng of RNA (1000 ng RNA in 10 μ L of DEPC H₂O and 2 μ L of bromophenol blue; see **Note 11**) on a 2% agarose gel containing ethidium bromide and perform electrophoresis at 100 V for 30–45 min to ensure band equality and RNA integrity (**Fig. 1**).

3.3.3. RT-PCR

3.3.3.1. RT REACTION

- Use **Table 1** to make an RT reagent cocktail, which is enough to run a sample for five different pairs of primers. Double the volume if a sample needs to be run for

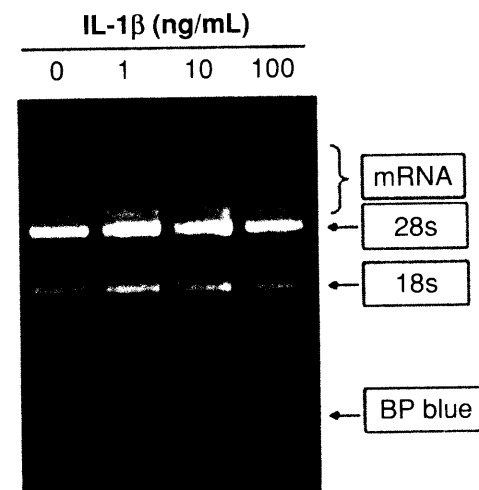


Fig. 1. RNA gel showing four RNA samples from HMC-1 stimulated with different concentrations of IL-1 β . RNA (1000 ng) was diluted in 10 μ L of DEPC water with 2 μ L of bromophenol blue added and electrophoresis conducted on a 2% agarose gel containing ethidium bromide. The messenger RNA (mRNA) shows a ladder pattern because of the differing lengths. The 28S and 18S bands of rRNA are shown.

- 6–10 pairs of primers. Additionally, increase the volume by multiplying the sample number. For example, there are five RNA samples to be run with three primers. Because there are only three primers, one serving (18 μ L of RT reagent cocktail) is required for each sample. For five RNA samples, we need 18 μ L $\times$ 5 = 90 μ L of RT reagent cocktail total. For better results, make one master mix of RT reagents, vortex, and distribute this to the five samples. To get enough RT reagent cocktail to distribute, add an extra serving (thus, 18 μ L $\times$ 6 = 108 μ L). Allow at least one extra sample for a housekeeping gene (**6,10,11**).
- Label one RT-PCR tube for every RNA sample. Each tube will receive 18 μ L of RT reagent cocktail and 1000 ng of RNA in 2 μ L of dH₂O for every serving.
- Centrifuge each tube for several seconds to collect the reagents and RNA to the bottom.
- Add tubes to a thermal cycler at 42°C for 20 min, 99°C for 10 min and 5°C for 5 min to transcribe cDNA.
- Remove tubes at this point or set thermal cycler for a 4°C soak until tubes can be removed.

3.3.3.2. PCR (SEE **NOTE 12**)

- While samples are being run through the RT cycle, prepare the PCR reagent cocktail according to **Table 2** (see **Note 13**). Multiply the volume of reagents by [(number of samples + 1 extra) $\times$ number of primers] and combine all the reagents in a master mix. Vortex the PCR reagent cocktail.

Table 1
Reverse Transcriptase Reagent Cocktail

Reagent	Volume
10x buffer	2 μ L
MgCl ₂ (25 mM)	4 μ L
dGTP (10 mM)	2 μ L
dATP (10 mM)	2 μ L
dCTP (10 mM)	2 μ L
dTTP (10 mM)	2 μ L
Oligonucleotide (50 μ M)	1 μ L
RNase inhibitor (20 U/ μ L)	1 μ L
Reverse transcriptase (50 U/ μ L)	1 μ L
dH ₂ O	1 μ L
RNA (1000 ng total) in dH ₂ O	2 μ L
Total	20 μ L

- Label the appropriate number of RT-PCR tubes and place them in a rack. Each tube will receive 2 μ L of the up primer, 2 μ L of the down primer (*see Note 14*; for equal RNA loading, at least one housekeeping gene primer should be added to your experiment, such as hypoxanthine phosphoribosyl transferase [HPRT]), 4 μ L of RT product (after it has been transcribed to cDNA), and 42 μ L of PCR reagent cocktail (**6,10,11**).
- Briefly centrifuge each tube to collect all materials to the bottom of the RT-PCR tube. If the thermal cycler requires samples to be covered with ultra pure mineral oil, do so at this time.
- Place tubes in thermal cycler and set temperature conditions and times according to primer specifications. Cycle number will depend on the amount of RNA present (usually 25–45 cycles is sufficient).
- When cycles are complete transfer sample to new tube, label, and freeze at -20°C .
- To visualize product, run 10 μ L of product with 2 μ L of bromophenol blue on a 2–3% agarose gel containing ethidium bromide at 100 V for 45–60 min (**Fig. 2**). Compare band lengths with a DNA ladder to ensure correct length of product. We use PhiX174 DNA/Hae III ladder (band sizes: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, and 118 basepairs).
- Cut bands and sequence if necessary.

3.3.4. Cytokine Measurement in Culture Supernatants Using ELISA

- Cytokine measurements are done using a commercially available ELISA kit, which requires 50–200 μ L of sample depending on the kit. Usually, 0.5-mL to 2-mL cultures in 6-, 12-, or 24-well tissue culture plates will provide enough supernatants for assays and generate enough product for detection (usually in the

Table 2
Polymerase Chain Reaction Reagent Cocktail

Reagent	Volume
10x buffer	4.8 μ L
MgCl ₂ (25 mM)	3.6 μ L
dGTP (10 mM)	0.8 μ L
dATP (10 mM)	0.8 μ L
dCTP (10 mM)	0.8 μ L
dTTP (10 mM)	0.8 μ L
T. aq polymerase (5 U/ μ L)	0.2 μ L
dH ₂ O	30.2 μ L
Total	42 μ L

pg/mL scale). Each ELISA kit comes with specific instructions and these should be followed very closely.

- A standard dilution is prepared from reagents provided in the kit.
- In a 96-well plate, add the standards and samples and incubate undisturbed for the indicated time.
- Wash the wells with wash buffer (provided) and add the appropriate conjugate antibody (also provided). Incubate undisturbed for the indicated time and wash again.
- A substrate solution should now be added which will change color if desired product is detected.
- After a short incubation period, add stop solution and run on a spectrophotometer at 450 nm with a correction at 570 nm.
- Generate a standard curve as shown in **Fig. 3**. Values from the ELISA will be interpolated or extrapolated from this curve.
- Take numbers from duplicate or triplicate samples and calculate a mean and standard deviation or standard error. Repeat experiments for reproducibility.
- Figure 4** shows the results of IL-8 production in IL-1 β activated HMC-1 cultures. Statistica for Windows (StatSoft, Tulsa, OK) is used for statistical analysis and Slidewrite Plus for Windows (Advanced Graphics Software, Encinitas, CA) for graph generation.

4. Notes

- To prepare phenol–chloroform: warm phenol to room temperature, place in 50°C waterbath to melt crystals. Pipet desired volume into appropriate sized flask. Add 8-hydroxyquinoline to a final concentration of 0.1%, and then add 1 mL of β -mercaptoethanol to every 100 mL of phenol. Then, add an equal volume of 0.5 M Tris-HCl (sterile, pH 8.0), cover with parafilm, and stir overnight. Aliquot phenol into sterile 50-mL tubes and add an equal volume of chloroform. Layer with 10 mL of 0.1 M Tris-HCl (pH 8.0) to prevent oxidation of the phenol. Wrap in foil to protect from light and store at 4°C .

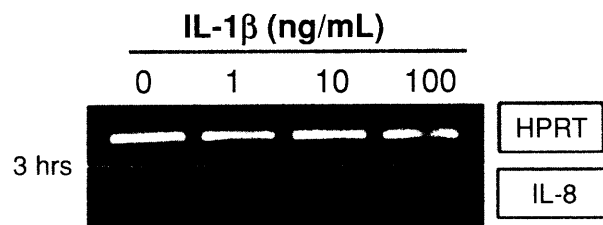


Fig. 2. RT-PCR gel showing expression of IL-8 in HMC-1. RNA from HMC-1 was subjected to RT-PCR with primers specific for human IL-8. Ten microliters of the PCR product with 2 μ L of bromophenol blue was electrophoresed on a 3% agarose gel. Hypoxanthine phosphoribosyl transferase (HPRT) was used as a housekeeping gene.

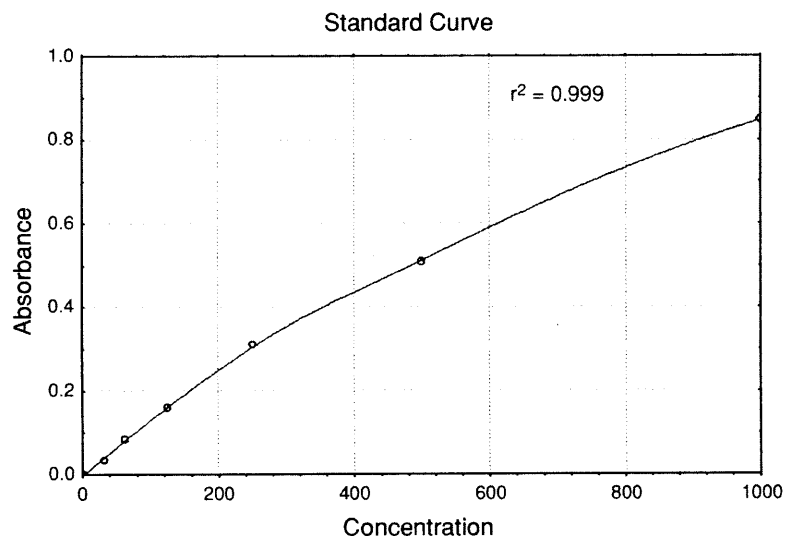


Fig. 3. Standard curve used for IL-8 ELISA analysis. A standard curve was constructed by plotting known concentrations of IL-8 on the x-axis against their respective absorbance at 450 nm corrected at 570 nm on the y-axis. The absorbance of the samples can be interpolated or extrapolated from this graph. Most computer software programs will calculate these values.

2. To increase yield of cells, rinse the flasks with 5 mL of PBS and add this residual cell suspension to the tube prior to centrifugation.
3. Leave one well empty to serve as a substrate blank. This substrate blank well should also be washed three times before addition of substrate.
4. Reading may be delayed for up to a maximum of 2 h at 18–25°C, if the plate is left in the dark and kept with its lid on in order to avoid evaporation.

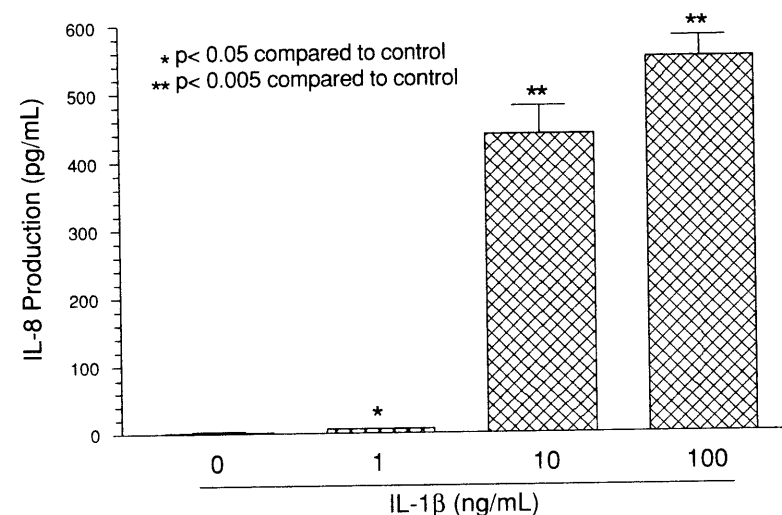


Fig. 4. A graph showing IL-8 production from HMC-1. Values taken from triplicate samples of HMC-1 treated with 1, 10, and 100 ng/mL of IL-1 β were used to generate a mean and standard deviation. The data were analyzed by Student's two-tailed *t*-test.

5. If the OD of the sample is out of the range of the standard curve, the sample needs to be either diluted with assay buffer (in case the OD is lower than that of the highest standard) or concentrated with Amicon filter (in case the OD is higher than that of the lowest standard). In the current experiment, the supernatants are diluted to 1:500 before the histamine assay.
6. The time at which the cell supernatants will be harvested is a factor for considering the cell concentration of cultures because mast cells proliferate quickly and begin to die when overcrowded. For cell supernatant harvests at 12 and 24 h, mast cell concentrations at 1×10^6 cells/mL work well and produce ample amounts of cytokine to be measured by ELISA. Experiments that require greater than 48 h incubation times work best with 0.5×10^6 cells/mL. Cell supernatant experiments should be performed in duplicate or triplicate and repeated for accuracy.
7. Sometime pretreatment of mast cells is required before activation with IL-1 β . If pretreatment lasting longer than 12 h (such as treatment with glucocorticoids or dominant-negative plasmids), the culture should be done in the tissue culture flasks. If pretreatments lasting less than 12 h (such as treatment with actinomycin or nuclear factor-kB inhibitor), the culture can be done in 6 well plates or tissue culture dishes. In both cases, cell viability should be checked before subject to preparation for RNA extraction. HMC-1 is a more resilient cell than CBDMC and is more likely to tolerate vortexing. If cell viability becomes a concern, slowly invert tube to mix mast cells and stimulants instead of vortexing before culturing in culture flask, plate, or dish.

8. It is better to leave part of the aqueous layer than to transfer any of the interface or RNA-Bee.
9. Depending on humidity, RNA pellet may not dry at room temperature. In this case, use a vacuum pump to help evaporate pellet. Drying the pellet too long may reduce its ability to be redissolved.
10. A 260:280 nm ratio of 2 is best for the RNA preparation (≥ 1.8 is acceptable; using 260 nm detects nucleic acids, whereas 280 nm detects proteins). A high 260:280 nm ratio (> 2.5) indicates phenol contamination, whereas a low 260:280 nm ratio indicates protein contamination. An absorbance at 230 nm indicates contamination with phenol or urea. An absorbance at 325 nm indicates particulate contamination or dirty cuvetes. If the RNA sample is contaminated with phenol, the phenol can be removed by the following procedure: (1) bring sample volume to 400 μL with 0.1% DEPC H_2O and mix; (2) add 500 μL of chloroform and vortex; (3) centrifuge at 12,000g for 5 min at 4°C; (4) remove aqueous layer with a pipet; (5) add 1/10 the volume with 3.0 M sodium acetate, pH 5.2, and mix; (6) double the sample volume with cold isopropanol and store overnight at -20°C; (7) centrifuge at 12,000g for 30 min at 4°C; (8) discard supernatant, add 1 mL of icecold 75% ethanol + 0.1% DEPC, and vortex (pellet should rise and fall intact); (9) centrifuge at 9,500g for 8 min at 4°C; (10) discard all liquid from RNA pellet and air dry at room temperature; (11) reconstitute RNA pellet with 20 μL of 0.1% DEPC H_2O ; and (12) characterize RNA on a spectrophotometer.
11. To make bromophenol blue, mix 0.25 g of bromophenol blue in 30 mL of glycerol and 70 mL of dH_2O .
12. Real-time PCR may be considered. The recent introduction of fluorescence-based techniques to PCR has allowed the development of real-time PCR, also called QPCR or kinetic PCR. Unlike conventional PCR that uses an endpoint analysis of the amplicon, real-time PCR is based on the detection and quantitation of the PCR product as it is made, using either fluorogenic specific probes or double-stranded DNA binding fluorescent dyes such as SYBR Green. During the exponential phase of the amplification, the fluorescent signal increase is directly proportional to the amount of PCR product. The real-time PCR technology is increasingly used, because it offers significant advantages such as high sensitivity, wide quantification range, and good reproducibility.
13. Depending on the salt concentration required for your specific primers, the MgCl_2 volume may need to be changed.
14. Better results are obtained when equal volumes of the up and down primer are mixed in a microcentrifuge tube, vortexed, and 4 μL of this mix is added to each tube. Primers were synthesized at the John Hopkins University School of Hygiene and Public Health DNA Synthesis Core Facility, Baltimore, MD.

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