Chapter 30

Production of Human and Murine Eosinophils In Vitro and Assay for Eosinophil Differentiation Factors

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1. Introduction

Bone marrow cells from a number of animal species have been used extensively in liquid and semisolid cultures to study hemopoiesis and to produce functional mature cells and factor-dependent cell lines (for review *see* ref. *1*). Neutrophils and macrophages are produced without added growth factors from murine long-term bone marrow cultures (*2*), while lymphoid cells (*3,4*) and megakaryocytes (reviewed in ref. *5*) can be induced under certain conditions.

When bone marrrow cells from mice or humans are established in tissue culture in the presence of eosinophil differentiation factor (EDF), mature functional eosinophils are produced and liberated into the non-

adherent cell population (*6,7*). This factor was originally proposed as interleukin-4 (*8*), but is now accepted as interleukin-5. Other names for IL5 are eosinophil colony stimulating factor (CSF-Eo), B-cell growth factor type II (BCGFII), and T-cell replacing factor (TRF).

Eosinophils are produced for only a relatively short time when marrow is cultured in the presence of IL5. This suggests that IL5 stimulates the differentiation of eosinophils from existing progenitor cells present in the marrow with no recruitment of eosinophil progenitor cells from stem cells. When the animal has a pronounced eosinophilia, subsequent culture of the marrow results in enhanced eosinophil production compared to cultures established from normal marrow. This is interpreted as an increase in eosinophil progenitors in the animal. We use the Helminth parasite *Mesocestoides corti* Hoeppli 1925 to effect such an increase in eosinophil progenitor numbers in the mouse. This parasite has been used extensively to study host/parasite interactions and causes a well-documented eosinophilia (*9*), with peak numbers of eosinophil progenitors appearing in the bone marrow 10 d after infection (*10*).

The assessment of eosinophil numbers from cultures can be done in one of two ways: (1) total cell counts (obtained from an electronic particle counter, e.g. Coulter counter, or by using a hemocytometer) and the percentage of eosinophils (obtained from a differential count on Giemsa-stained smear or cytocentrifuge preparation) are used to calculate the number of eosinophils, or (2) by assaying for eosinophil peroxidase, which can be related to eosinophil numbers (*11*).

A microplate modification of the culture system (*12*) provides a method for assaying sources of IL5 since there is as yet no IL5-dependent cell line as there are for IL2 and IL3. This IL5 assay is based on assessment of eosinophil numbers by quantifying eosinophil peroxidase in microplate bone marrow cultures that have been incubated with IL5 samples. This assay also detects the eosinophil differentiation activity of GM-CSF and IL3. The B cell activity of mouse IL5 can be measured using the BCL_1 cell line (*8*) though this assay also detects interleukin-4 (B-cell-stimulatory factor [BSF1]).

2. Materials

1. Parasite: The second stage larvae (tetrathyridia) of the Cyclophyllidean Cestode *M. corti* are maintained by intraperitoneal passage in mice, where it reproduces itself vegetatively. The larvae can be stored in Dulbecco's Phosphate Buffered Saline "A" (PBS) at 4°C for several weeks, and for longer periods if fetal calf serum is added (*13*). To our knowledge there have been no recorded cases of human infections

with *M. corti*, although there have been several cases of infection by other species of *Mesocestoides* (*14,15*). Parasites for reinfecting mice are harvested from mice that have been infected for several weeks. The only noticeable visible effect of the infection is an increasing abdominal distension. It may be necessary to occasionally passage the parasite into another strain of mouse (e.g., CBA) as it seems to lose the ability to stimulate high eosinophilia after several months passage through the same strain. For availability of the parasite, refer to "International Register of Living Helminth Species and Strains," published by WHO; or "Register of Parasitic Protozoa, Helminths, and Arthropods of Medical and Veterinary Importance," produced by the British Society for Parasitology.

2. Mice: We routinely use Balb/c.nimr mice, 6–8 wk-old, which are maintained under SPF conditions and are allowed free access to food and water. Any infections the mice contract before the marrow is harvested for culture may result in changes in the cell numbers and types seen in the marrow cultures.
3. Media: The basic medium used is RPMI 1640 purchased in powder form and reconstituted as recommended by the manufacturer. We use two basic forms of RPMI as indicated below:

	(1) HEPES Medium	(2) Culture Medium
HEPES buffer	20 m*M*	10 m*M*
Sodium bicarbonate	None	24 m*M*
Glutamine	2 m*M*	2 m*M*
Sodium pyruvate	None	1 m*M*
Penicillin	100 U/mL	100 U/mL
Streptomycin	100 μg/mL	100 μg/mL
Monothioglycerol (Sigma)	None	75 μ*M*

The above media are stable at 4°C for several months. Glutamine is unstable, so fresh glutamine is added from a frozen (–20°C) 200 m*M* stock solution and the medium used within 2 wk. Glutamine is a general requirement in tissue culture medium and has been found to be necessary for hemopoietic cell differentiation (*16*).

The media are further supplemented and used as follows:

a. Bench medium: HEPES medium is supplemented with newborn calf serum to 5% v/v. This is used for cell and tissue collections and preparation, and is stored at 4°C. It has the advantage over bicarbonate buffered medium that it does not change its pH while outside a CO_2 environment.

b. Bone marrow culture medium; Culture medium is supplemented with $10^{-6}M$ hydrocortisone and 15% fetal calf serum. The pH of this medium is maintained by culturing in an atmosphere of 5% CO_2 in air.

(1) Media additives:

(a) Hydrocortisone: A $10^{-2}M$ (48.45 mg/mL) stock solution of hydrocortisone sodium succinate in PBS is filter sterilized (0.22 μm pore) and stored in 50–100 μL aliquots at –20°C. 10 μL stock solution is added to 100 mL bone marrow culture medium. Any remaining stock hydrocortisone solution is not refrozen, but discarded.

(b) Fetal calf serum: This has to be selected for optimal eosinophil growth, since some batches result in negligible eosinophil production in cultures. Whether this is caused by inhibitors or lack of growth factors is not known. It is preferable to test the fetal calf serum in both the EDF assay and in long-term cultures.

(c) Gentamicin: A stock solution of 5 mg/mL gentamicin sulfate in PBS is filter sterilized, stored at –20°C, and diluted 1:100 into medium for use. Gentamicin is occasionally required when assaying column fractions for IL5.

4. Sources of Interleukin-5: Native murine IL5 can be obtained from mitogen-or antigen-stimulated spleen cells (*11*), T cell clones (*17*), or the EL4 lymphoma cell line (*18*). However, these sources also contain other lymphokines. The T cell hybrid NIMP-TH1 produces IL5 in the apparent absence of other known lymphokines (*19*) and has provided the most useful source of IL5 until recombinant material from transfected monkey COS cells became available (*20*). There is no known source of native human IL5 and work on human eosinophils has been based on the cross reactivity of murine IL5 (*21*). However, recombinant human IL5 is now available from transfected COS cells (22). IL5 has also been detected in the serum of parasitized animals (*8*), and recent work has demonstrated the presence of a factor stimulating human eosinophil differentiation in serum from patients undergoing eosinophilia (23).

Interleukin-3 and GM-CSF have some eosinophil differentiation activity (*6*) so limited number of eosinophils can be produced in cultures using WEHI-3 conditioned medium or commercially available GM-CSF. When sources of IL5 such as crude spleen conditioned medium that contain other lymphokines as contaminants are used, large numbers of neutrophils and/or macrophages are produced so that eosinophils represent only a small percentage of the nonadherent cells.

Methods detailing production of the above sources of IL5 are given in ref. 24.

5. Eosinophil cultures: A Class II Microbiological safety cabinet is required for human cultures, and all waste materials should be autoclaved. Murine cultures are established under standard tissue culture conditions. A gassed 37°C incubator is required, which has to be humidified for agar, cluster, and microplate cultures.
   a. Agar cultures: We use Difco Bacto-Agar that is preselected for colony growth. A 5% w/v stock solution is prepared by suspending the agar in distilled water and placing in a boiling waterbath for 5 min. The agar is stored at room temperature. We do not autoclave the agar since this seems to introduce some toxicity into the system. Leukocyte migration plates (Sterilin, Teddington) are used in place of Petri dishes as this allows the whole agar culture to be easily recovered and stained for assessing colony number and type. The small culture volume (400 µL) results in savings in materials and reagents. A square 100-m*M* Petri dish is used as the container for the leukocyte migration plate.
   b. Long-term cultures: These cultures are established in either flasks or Cluster plates. We use 25–80 cm^2 flasks from which large numbers of eosinophils can be produced for functional studies. Most experimental work is done using 24-well Cluster plates. A cytocentrifuge is useful to prepare slides for staining and differential counts, the morphology being clearer than on smears.
   c. Microplate cultures: The cultures are established in 96-well round (U) bottomed microtiter plates. The flat bottomed 96-well microtiter plates are not satisfactory for this assay system.
6. Eosinophil Peroxidase Assay
   a. Peroxidase buffer: 0.05*M* Tris-HCl pH 8.0. Filtered (0.45 µm pore) and stored at room temperature.
   b. *o*-Phenylenediamine (OPD): a stock solution of 10 mg/mL in distilled water is stored in 1 mL aliquots at –70°C, where it is stable for several months.
   c. 30% w/v hydrogen peroxide stored at 4°C.
   d. Triton X-100: a 10% stock solution in water is stored at 4°C.
   e. 4*M* sulfuric acid.
   f. Complete substrate solution: to 48.5 ml peroxidase buffer add 1 mL OPD stock solution, 0.5 mL Triton X-100 and 6 µL hydrogen peroxide. OPD is light sensitive and so this solution

should not be prepared until it is required. An automatic microplate reader with a 490-nm filter is recommended for reading the plates.

7. Eosinophil Stains
   a. Giemsa: Buffer; Sorensens buffer concentrate pH 6.8, diluted to 3.3 m*M* in distilled water. Giemsa (10%) stock solution is diluted 1:5 with buffer for use.
   b. Congo Red: Dissolve 5 g Congo Red in 50 mL distilled water then add 50 mL ethanol. The solution is stable and can be reused. Different batches of Congo Red seem to vary in their ability to stain eosinophils.
   c. Toluidine Blue: Add 1 g Toluidine Blue to 100 mL methanol. Acidify by adding 5 mL 2*M* HCl. The solution is stable and can be reused.
   d. Luxol-Fast-Blue: Add urea to 70% ethanol until saturated (approximately 250 g/L), then filter through a Whatman No. 1 filter paper. Dissolve 1 g Luxol-Fast-Blue in 100 mL of urea saturated 70% ethanol. The solution is stable and can be reused.
   e. Harris' Hematoxylin: Dissolve 1 g hematoxylin in 50 mL ethanol, and 100 g aluminum ammonium sulfate (or aluminium potassium sulfate) in 1 L distilled water (with gentle heating). Add the hematoxylin solution to the salt solution and bring to the boil rapidly. CAREFULLY add 2.5 g mercuric oxide (a violent reaction may occur if added too rapidly) and allow to cool. Filter. Add 4 mL glacial acetic acid to each 100 mL of stain and store at room temperature. The stain can be reused extensively.

3. Methods

3.1. Parasite Passage

With the parasite in a plastic Universal tube in PBS:

1. Aspirate 100–200 µL of PBS into a 1 mL syringe and expel the air bubble.
2. Allow the parasites to settle at unit gravity.
3. Insert the end of the syringe into the parasite pellet and fill the syringe.
4. Invert the syringe and allow the parasites to settle. Expel excess PBS and refill with more parasites if necessary.
5. Fit a 0.8 x 40 mm needle and expel air and excess PBS.
6. Inject 100 µL parasite ip into each mouse.

3.2. Parasite Harvest

1. Kill the mouse by cervical dislocation.
2. Cut the abdominal skin and expose peritoneal wall.
3. Using a 5 or 10 mL syringe with a 0.8 x 40-mm needle inject 5–10 mL of PBS into the peritoneum.
4. Withdraw the parasite/PBS into the syringe.
5. Expel parasite/PBS into a sterile plastic Universal bottle.
6. Wash the parasites several times by filling the bottle with PBS, allow the parasites to settle, and pour off supernatant.
7. Use the parasites to infect more mice (*see* Section 1) and store the remainder at 4°C.

3.3. Marrow Collection and Cell Preparation

3.3.1. *Mouse Marrow* (See *Note 1*)

1. Fill a suitable container with 70% ethanol and immerse a pair of scissors and forceps. Use a tissue to dry the instruments before use and replace them into the alcohol between each procedure.
2. Kill mice by cervical dislocation.
3. Fill a 5-mL syringe with bench medium and fit a 0.4 x 12-mm needle. We use the syringe type, which is packed in an outer polypropylene case, the case being used as a sterile "home" during the procedures.
4. Wet the animal's fur with 70% ethanol and place the animal on its back.
5. Pull up the abdominal skin with forceps and make a cut across the abdomen with the scissors held vertically.
6. Pinch the skin on each side of the cut between thumb and forefinger, and enlarge the incision by pulling the skin toward the head and tail. The incision should enlarge around the animal and the skin finally break at the animal's back. Continue to pull the skin back toward and over the tail and legs until the muscles of the upper and lower legs are exposed.
7. Complete the skinning by grasping the tibia/fibula with forceps and the skin with the other hand and pulling the leg from the skin until the foot is clear and the whole leg completely skinned.
8. Remove the legs from the animal by a combined movement that both dislocates the leg from the pelvis and cuts the muscles, ideally leaving the leg complete with femoral head.
9. Holding the femur with forceps, cut across the femur with the scissors to remove the head.
10. Flush out the marrow by holding the femur with forceps (foot held

upwards) and inserting the needle as far as possible up into the femur, being careful not to put the needle through the knee joint. With the needle in the femur, bend the needle to an angle of about 45° to prevent the cells running down the outside of the needle and onto the syringe. Position the leg above the collection tube and when the medium is injected into the femur the cells will be flushed out and drip into the tube. The cells often congregate as a lump at the end of the bone, so withdraw the needle and touch the cut end of the femur onto the tube and dislodge the cells using the syringe needle and flush them into the tube. Pool the marrow from several mice into one tube.

11. Centrifuge the cells (300*g* for 8 min) and remove the supernatant.
12. Resuspend the cells in 5 mL of medium and break up any large cell clumps by repeated aspiration into a 10 mL pipet. If the cells are not to be established in culture immediately, then resuspend them in bench medium; otherwise use bone marrow culture medium.
13. Aspirate the cells into a 10 mL syringe via a 9.8 x 40-mm needle and expel them back into a tube through a 0.4 x 12-mm needle.
14. Count the cells and adjust to the required density.
15. A differential count can be done to assess the degree of marrow eosinophilia (*see* Note 2).

3.3.2. Human Marrow

Marrow can be obtained from aspiration of the iliac crest or the sternum (during cardiothoracic surgery) or from ribs removed during thoracotomy. Use of marrow samples for research purposes requires the informed consent of the patient and the approval of the local Hospital Ethics Committee.

1. Resuspend marrow in bench medium to 1–2 x 10^7 cells/mL.
2. Layer 10 mL cells onto 10 mL Ficoll-Paque in Universal tubes.
3. Centrifuge at 600*g* for 35 min at room temperature.
4. Collect the interface cells (mononuclear cells) and add bench medium.
5. Centrifuge (600*g* for 10 min) and resuspend pellet in bench medium.
6. Centrifuge (500*g* for 10 min) and resuspend pellet in bone marrow culture medium at 10^6 cells/mL.

Approximately 0.5–1.5 x 10^7 mononuclear cells are obtained from 1mL of normal marrow aspirate.

3.4. Eosinophil Cultures

3.4.1. Murine Microplate Cultures

1. Adjust the marrow cell concentration to 1 x 10^6 cells/mL in bone marrow medium.

2. Aliquot 10 µL of sample (or sample dilutions if titrating) into duplicate microplate wells.
3. Add 100 µL cells/well (including several wells without IL5 to use as control cultures) and incubate in a humidified atmosphere at 37°C and 5% CO_2 in air.
4. After 5 d assay the cultures for eosinophils by either total cell counts and differential counts or by peroxidase (*see* Section 3.7).

3.4.2. Human Microplate Cultures

1. Set up cultures as for mouse microplate cultures (steps 1–3 above).
4. Every 7–10 d carefully aspirate 50 µL of medium and replace with fresh medium containing sample.
5. After 21–28 d in culture, determine total cell numbers (Coulter counter or hemocytometer) and perform differential cell counts on a cytocentrifuge or smear preparation, or perform a peroxidase assay.

3.4.3. Agar Cultures

1. Melt the agar in a boiling waterbath and hold at 45°C.
2. Adjust the bone marrow cells to 2×10^5 cells/mL (or dilutions of cells if required) in bone marrow medium and hold in a 37°C waterbath.
3. Aliquot 40 µL of IL5 or other growth factors or their dilutions into each well of a leukocyte migration plate.
4. Pipet 1 vol of agar into 15 vol of the cell suspension, mix well, and immediately aliquot 400 µL/well.
5. Place the plate into the upturned lid of a 100-mm-square Petri dish containing a moist piece of tissue or filter paper and cover with the square Petri dish base.
6. Allow the agar to solidify, putting the plates for a short time at 4°C if the laboratory temperature is high.
7. Place in a humidified incubator at 37°C in 5% CO_2 in air.
8. Fix and stain mouse cultures after 5–7 d, and human cultures after 14–21 d (Sections *3.5,3.6*).

3.4.4. Long-Term Cultures (See Note 6)

1. Adjust the bone marrow cells to 1.5×10^6 cells/mL. If large numbers of eosinophils are required within 1-wk use marrow from parasitized animals, 9–20 d postinfection.
2. Aliquot the cells into flasks or Cluster plates, 10 mL of cells for a 25 cm^2 flask, 30 mL for an 80 cm^2 flask or 1 mL/well for 24-well cluster plates.
3. Add IL5 at a predetermined optimal dilution and place in a 37°C incubator in 5% CO_2 in air. The Cluster plates will require a humidified incubator.

4. Every 6–7 d, gently tap the cultures to dislodge the nonadherent cells and remove all the supernatant and nonadherent cells.
5. Add fresh medium containing IL5 and place back into the incubator.
6. Determine the total cell number of nonadherent cells and prepare a smear or cytocentrifuge preparation.

3.5. Fixing the Agar Cultures

We find it necessary to stain the cultures to accurately count eosinophil colonies since assessing the cultures by morphology alone under the inverted microscope is misleading—we have found both tight and very loose colonies of eosinophils. Mouse cultures are stained using Congo Red and counterstained with either Harris Hematoxylin or Toluidine Blue. We have had little success using Luxol-Fast-Blue for staining mouse agar cultures. Human cultures can be stained with any of the above stains including Luxol-Fast-Blue. Colonies are defined as clusters containing more than 40 cells.

1. Set the leukocyte migration plate at an angle of about 30° in a retort stand. With a slide held horizontally and resting against the rim of a well, direct the agar disk onto the slide by flushing with a stream of PBS from a wash bottle. It is possible to mount three agar disks on one standard 26 x 76 mm microscope slide.
2. Cover the agar disk(s) with a piece of dry Whatman No. 1 filter paper and put onto a warm plate to dry. Do not have the warm plate too hot.
3. Remove the filter paper just before it is completely dry and allow the agar disk to dry completely.
4. Fix in fresh methanol for 15 min before staining (Section 3.6.).

3.6. Eosinophil Stains

3.6.1. Harris Hematoxylin and Congo Red

1. Place the fixed slide in acidified Harris Hematoxylin for 5 min.
2. Wash under gently running tapwater until blued.
3. Place in Congo Red and stain for 15 min.
4. Wash briefly under gently running tapwater and dry.
5. Nuclei stain blue, eosinophil granules red.

3.6.2. Congo Red and Toluidine Blue

1. Stain the fixed slide for 10 min in Congo Red.
2. Wash in 50% ethanol for 5 min.
3. Stain in Toluidine Blue for 5 min.

4. Rinse in water and dry.
5. Eosinophil granules stain reddish-brown, mast cell granules dark blue. Neutrophils and macrophages should not have any cytoplasmic staining.

3.6.3. Luxol-Fast-Blue

1. Stain the fixed slide for 1.5 h in Luxol-Fast-Blue.
2. Wash under gently running tapwater for 3 min.
3. Dry.
4. Stain with Harris Hematoxylin for 2 min (30 s for cytocentrifuge preps).
5. Wash under gently running tapwater for 3 min.
6. Nuclei stain blue, eosinophils have green granules.

3.7. Eosinophil Peroxidase (EPO) Assay (See *Note 4*)

1. Aspirate most of the medium from the bone marrow cultures, taking care not to suck out the nonadherent cells of the pellet. The medium does not interfere with the assay, but the wells become overfilled if it is not removed.
2. Add 100 µL of substrate solution to each well and leave at room temperature for 30 min. It is not necessary to incubate in the dark for this short period.
3. Stop the reaction by the addition of 50 µL of 4*M* sulfuric acid.
4. Determine the absorbance at 490 nm. Once the acid has been added the color is stable for several hours.

3.8. Fixing and Staining Cytocentrifuge Preparations

1. Ensure the cytocentrifuge preparation (or smear) is dry then fix in methanol for 5 min.
2. Stain the fixed slide in Giemsa for 2 min.
3. Wash under tapwater.
4. Blot dry.
5. Examine with oil immersion, a green Kodak Wratten filter No. 11 may assist in identifying eosinophils.
6. Nuclei are reddish-purple, eosinophils have red to orange granules. Basophilic granules are blue. The eosinophil stains in Section 3.6. can be used instead of Giemsa for staining cytocentrifuge preparations and smears.

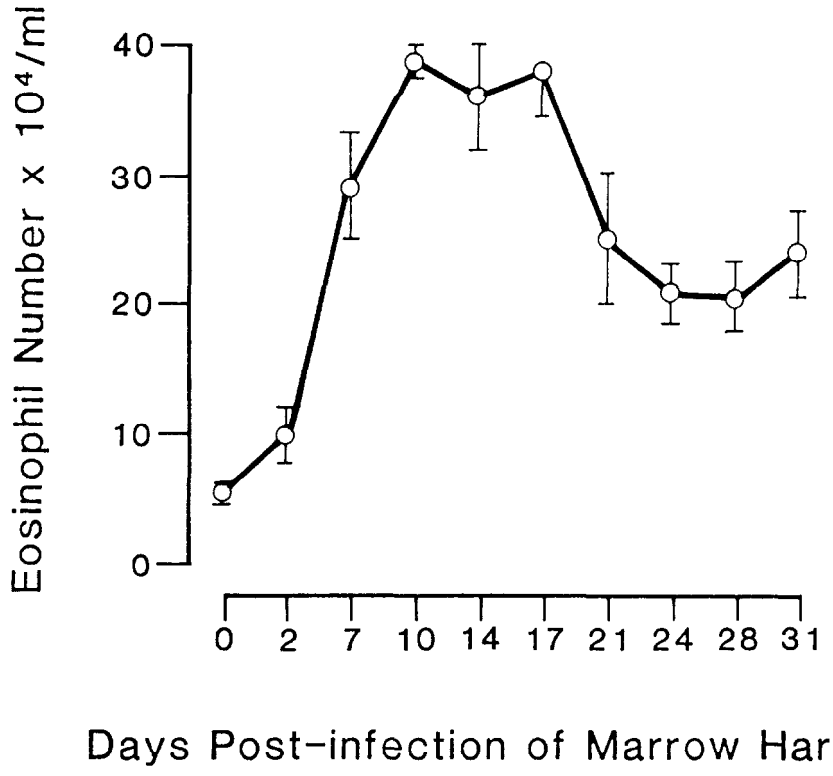

Fig. 1. Number of eosinophils in supernatant from murine bone marrow cultures after 7 d incubation. Marrow was harvested from mice on different days post-infection with *M. corti*. All cultures were established in the presence of IL5 from a T-cell clone conditioned medium. Eosinophil numbers represent the mean ± 1 standard deviation of three replicate cultures.

4. Notes

1. When taking bone marrow we work in the open laboratory, using alcohol dried instruments. There are very few problems with contamination at this stage. The animal's fur is wetted with alcohol to prevent possible contamination by the fur being flicked.
2. The importance of using parasitized mouse marrow for eosinophil production is illustrated in Fig. 1. It shows the production of eosinophils after 7 d incubation using marrow harvested from mice on different days after infection. Uninfected mice usually produce relatively few eosinophils. We generally use marrow from mice infected for 9–20 d.
3. Use of donor horse serum (DHS) in long term cultures: If a good batch of fetal calf serum has been obtained, we do not find it necessary to

Table 1
The Production of Eosinophils in the Presence and Absence of Hydrocortisone and Donor Horse Serum

Medium additives[a]			Days in culture				
DHS	Hc		7	14	21	28	35
+	+	(a)[b]	35.90	41.94	42.3	42.33	42.34
		(b)	58 ± 9	43 ± 6	8 ± 4	0.5 ± 1	0.2 ± 0.4
+	–	(a)	27.10	37.50	38.61	38.63	38.64
		(b)	41 ± 10	33 ± 4	7 ± 4	0.3 ± 0.5	0.2 ± 0.1
–	+	(a)	46.50	54.00	54.84	54.96	55.02
		(b)	63 ± 5	38 ± 12	11 ± 5	2 ± 3	1 ± 1
–	–	(a)	52.50	53.19	54.26	54.30	54.30
		(b)	64 ± 7	32 ± 18	8 ± 3	1 ± 1	0 ± 0

[a](+) indicates medium supplemented with 5% donor horse serum (DHS) or hydrocortisone (Hc), (–) indicates absence from medium.

[b](a) = Cumulative total of eosinophils x 10^4/mL. (b) = eosinophils as percent of total nonadherent cells (mean ± 1 standard deviation of six duplicate cultures). Bone marrow from parasitized mice was incubated in the presence of T-cell clone conditioned medium.

include DHS in the bone marrow culture medium. Table 1 shows the cumulative numbers of eosinophils produced over 35 d in culture together with the percent of nonadherent cells that are eosinophils. It can be seen from Table 1 that the presence of DHS suppresses eosinophil numbers, though the percentage of nonadherent cells that are eosinophils is increased because of reductions in the other cell types. Hydrocortisone has a short-term suppressive effect in cultures without DHS, but is beneficial for the longer term cultures.

4. The short-term microplate culture system is used to assay IL5 samples, to find the optimal amount of IL5 to produce eosinophils in the longer-term cultures, and to test fetal calf serum batches. In assaying for IL5 in column fractions, it is advisable to include gentamicin in the bone marrow culture medium. Use marrow from mice infected for between 9–20 d for establishing the cultures. Cells of the neutrophil lineage constitute 30–60% of total normal human marrow cells and, in the absence of a suitable growth factor, such cells do not survive in the microplate cultures for longer than 21 d. Since the eosinophil perioxdase (EPO) assay detects human myeloperoxidase (even in the presence of the enzyme's inhibitors such as KCN), the assay is only suitable for use in the human microplate culture system after 21 d culture with sam-

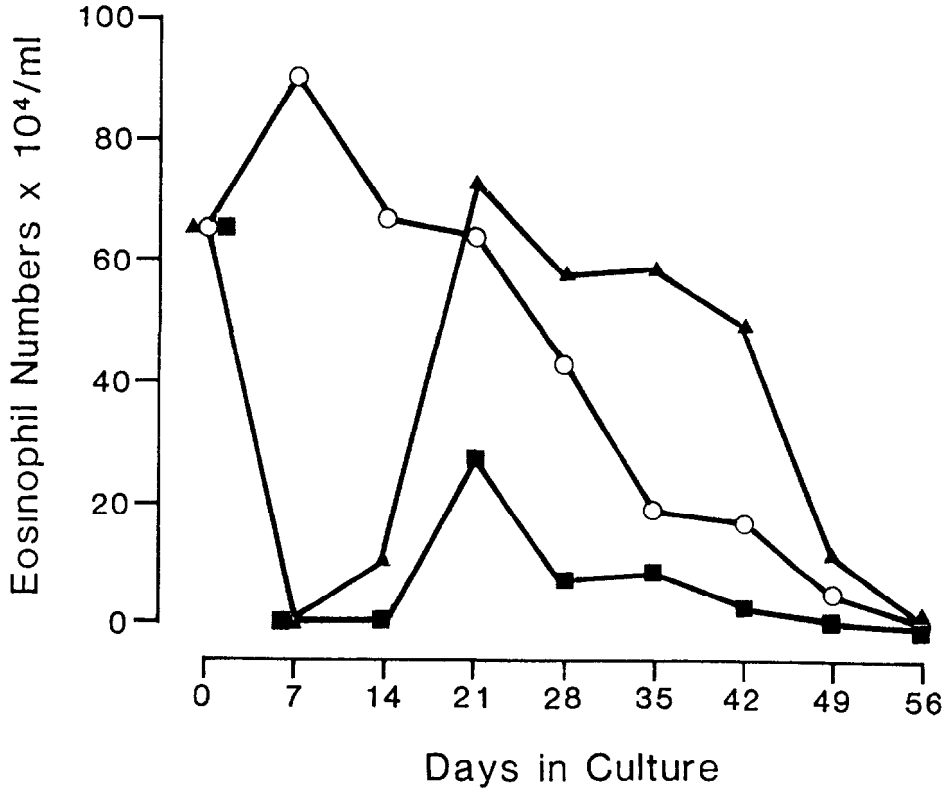

Fig. 2. Production of eosinophils from bone marrow cultures established from *M. corti* infected mice. Cultures were incubated continuously in the presence of NIMP-TH1 conditioned medium from day 0 of culture (○); day 7 of culture (▲); and day 14 of culture (■). Eosinophil numbers represent the mean ± 1 standard deviation of three replicate cultures.

ples that are known not to contain neutrophil growth factors. Figure 2 shows the production of human eosinophils (and total cells) from microplate bone marrow cultures stimulated with NIMP-TH1 conditioned medium. Samples that are cytotoxic and kill the marrow inoculum may appear positive in the peroxidase assay, since eosinophil peroxidase has not been degraded during the culture period. For this reason it is prudent to examine the cultures before assaying for EPO, and wells that have no visible cell pellet noted.

5. Agar Cultures: Some workers use slides pre-coated with 0.3% agar to mount their agar disks on. This is to ensure that the agar sticks to the slide and is not pulled off when the filter paper is removed.
6. Figure 3 shows the time course of eosinophil production of cultures established in the presence of NIMP-TH1 conditioned medium. The

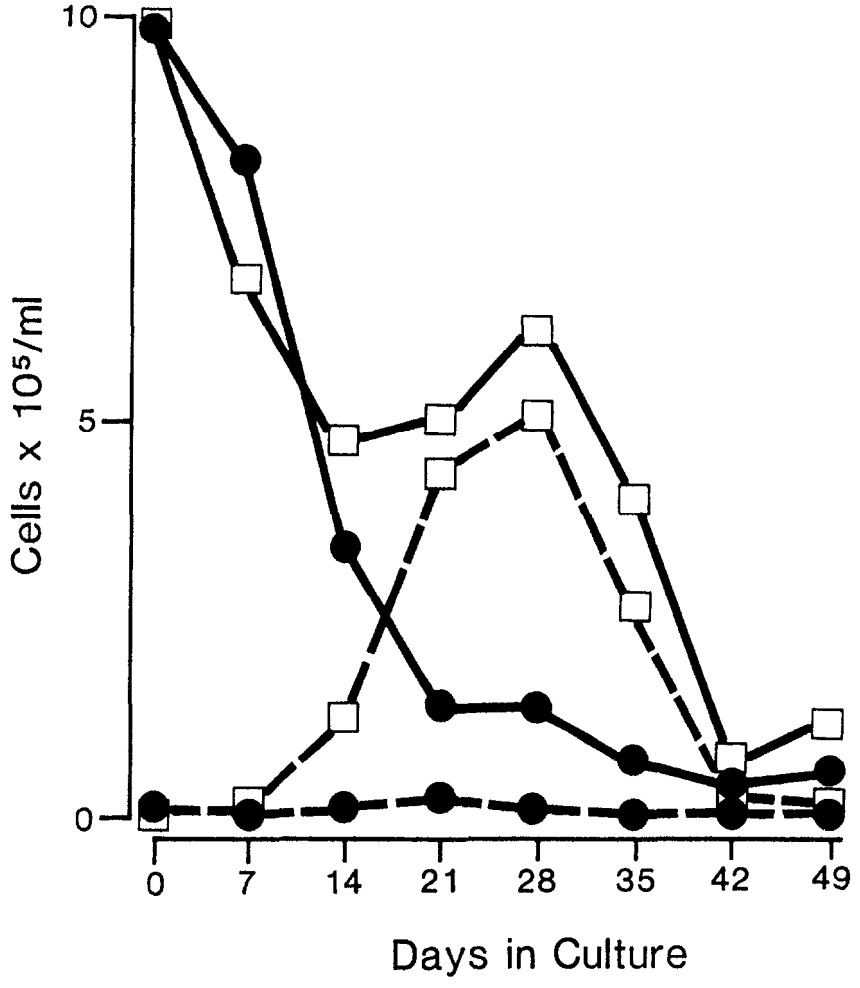

Fig. 3. Production of total cells (—) and eosinophils (- - -) from microwell cultures of normal human bone marrow established in the presence (□) and absence (●) of murine EDF (NIMP-TH1 conditioned medium).

marrow was harvested from mice infected for 14 d with *M. corti* with a marrow eosinophilia of 45%. When IL5 was added from day 0 of culture, most of the eosinophils were produced in the first 2–3 weeks when over 80% of the nonadherent cells were eosinophils (85.7 ± 5.1% at 7 d and 83.7 ± 1.2% at 14 d. Without IL5 the eosinophils present in the marrow samples disappear at the end of 7 d, and addition of IL5 for the first time at this point ensures that subsequent eosinophils are produced in vitro from the precursors and are not surviving eosinophils from the inoculum. In cultures that have the addition of IL5 de-

Table 2
Number of Eosinophils Produced from Mouse Bone Marrow After 7 d Incubation in the Presence of IL5

EDF dilution	Source of IL5			
	NIMP-TH1		rmIL5	
	Number[a]	%	Number[a]	%
1:100	63.36 ± 6.99	52.2 ± 5.1	nd	
1:200	nd		20.52 ± 1.89	25.9 ± 3.0
1:300	22.00 ± 0.40	28.0 ± 2.6	nd	
1:400	nd		18.49 ± 0.54	21.4 ± 4.8
1:1000	3.95 ± 0.30	7.0 ± 1.1	22.60 ± 1.56	34.3 ± 1.8
1:3000	0.76 ± 0.17	1.9 ± 0.1	10.14 ± 0.65	14.3 ± 0.1
none	0.11 ± 0.15	0.2 ± 0.3		

[a]Number given as eosinophils x 10^4/mL. Both number and percent given as mean ± 1 standard deviation of 4 replicate cultures.

layed for 7 d, it takes another 14 d before peak eosinophil numbers are produced, when the eosinophils may represent up to 90% of nonadherent cells. Fewer eosinophils are produced if the addition of IL5 to the cultures is delayed for 14 d, when at peak production time the eosinophils only represent 27% of supernatant cells.

Recombinant mouse IL5 (rmIL5) is active in stimulating the production of eosinophils in murine marrow cultures. Table 2 shows the effect of different concentrations of rmIL5 and NIMP-TH1 conditioned medium on eosinophil production after 7 d culture.

It must be noted that eosinophil production is very variable between experiments, as well as having large variations between "replicate" cultures. This variation between replicates tends to increase as the cultures get older, particularly in Cluster plate cultures. This may represent cloning of the very young committed eosinophil progenitors that are present in the marrow at a low frequency.

A total lack of eosinophil production in the presence of a known IL5 sample may be due to an unsatisfactory batch of fetal calf serum, or a fault in the medium or its preparation.

7. Other sources of eosinophil progenitors: Spleen and fetal liver are focal points of hemopoiesis. We have no experience with fetal liver, but spleen cells have been used in agar cultures and in the IL5 assay. Uninfected mouse spleen cells produced no eosinophil colonies when incubated in agar in the presence of EL4 conditioned medium, but spleen cells from mice infected for 12 d do produce eosinophil colonies. Cells from the same spleen gave a positive assay result when

used in the IL5 assay, but had to be partially purified and the EPO level above background (i.e., unstimulated cultures), was not as great as marrow cells.

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