

REGULATION OF HUMAN EOSINOPHIL VIABILITY, DENSITY,  
AND FUNCTION BY GRANULOCYTE/MACROPHAGE  
COLONY-STIMULATING FACTOR IN THE  
PRESENCE OF 3T3 FIBROBLASTS

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Eosinophils, cells that are associated with asthma (1, 2), helminthic infections (3), and other clinical disorders, are thought to reside in the peripheral blood for <12 h and in connective tissues for several days (4). The in vitro survival of human peripheral blood eosinophils is limited to <2 d. Because the eosinophilia that normally accompanies helminthic infections is suppressed in athymic mice (5) and in normal rats or mice that have been depleted of their T lymphocytes (6–10), it has been concluded that eosinophilopoiesis is modulated by T cell factors. Granulocyte/macrophage colony-stimulating factor (GM-CSF)<sup>1</sup> is thought to be one of the T cell-derived factors that regulates eosinophils in vivo. Upon intravenous infusion in primates, GM-CSF is known to induce granulocytosis with eosinophilia (11), and will induce human bone marrow progenitors to form colonies of eosinophils in vitro (12). Mature peripheral blood eosinophils may possess high-affinity GM-CSF receptors (13), and recent studies (14) have demonstrated that the short-term in vitro exposure of human eosinophils to biosynthetic (recombinant) human GM-CSF (rH GM-CSF) augments their antibody-dependent killing of *Schistosoma mansoni* larvae and their calcium ionophore A23187-stimulated generation of leukotriene C<sub>4</sub> (LTC<sub>4</sub>). Hypodense eosinophils, defined by their lower-than-normal densities in discontinuous metrizamide or Percoll gradients, have been isolated from patients with idiopathic hypereosinophilic syndromes or from hosts chronically infected with *S. mansoni*. These less

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<sup>1</sup> *Abbreviations used in this paper:* GM-CSF, granulocyte/macrophage colony-stimulating factor; rH GM-CSF, biosynthetic (recombinant) human GM-CSF; MEM, Eagle's minimal essential media; LTC<sub>4</sub>, leukotriene C<sub>4</sub>.

dense eosinophils exhibit enhanced antibody-dependent cytotoxicity (15), and generate more LTC<sub>4</sub> when stimulated with calcium ionophore (16), suggesting that this hypodense phenotype may be important in helminthic infections. We now report that normodense human peripheral blood eosinophils cultured with rH GM-CSF in the presence of mouse 3T3 fibroblasts survive for at least 14 d. The resulting eosinophils become hypodense and have an augmented capacity to generate LTC<sub>4</sub> and to kill *S. mansoni* larvae.

### Materials and Methods

*Isolation of Eosinophil- and Neutrophil-enriched Cell Preparations.* Human eosinophils and neutrophils were isolated using the technique of Vadas et al. (17) modified by Owen et al. (18) from the peripheral blood of seven different donors, none of whom were ingesting aspirin, other nonsteroidal antiinflammatory drugs, or corticosteroids. Three of these donors had no diagnosed clinical disorder and had normal white blood counts and differentials, whereas a fourth donor had 12% eosinophilia. The other three donors were diagnosed as having allergic rhinitis, allergic conjunctivitis, and/or asthma, and 2–5% of their white blood cells were eosinophils. Whole blood (45-ml aliquots) was aspirated into 60-ml syringes, each containing 1,000 U of sterile heparin (Sigma Chemical Co., St. Louis, MO) in HBSS. All subsequent steps were performed under sterile conditions in a laminar flow hood using reagents that were filtered through 0.45- $\mu$ M filters. To each syringe we added 9 ml of prewarmed PBS containing 4.5% (wt/vol) of 150,000–200,000 *M<sub>r</sub>* dextran (BDH Chemicals, Poole, England), pH 7.3. After a 45-min incubation at 37°C, the buffy coats were collected, an equal volume of enriched MEM (Eagle's modified essential medium containing 100 U/ml of penicillin G, 100  $\mu$ g/ml of streptomycin, 2 mM L-glutamine, and 1 mM Hepes [Gibco Laboratories, Grand Island, NY]) was added along with 45 U/ml of deoxyribonuclease 1 (Sigma Chemical Co.), and the cells were centrifuged at 250 *g* for 10 min at 4°C. The mixed leukocyte preparations were resuspended in enriched MEM containing 10% FCS (Gibco Laboratories) at a density of  $3.3 \times 10^7$  cells/ml.

Metrizamide at concentrations of 18, 20, 21, 22, 23, and 24% (wt/vol) (Nyegaard and Co., Oslo, Norway) was dissolved in Tyrode's buffer, pH 7.2, containing 0.1% gelatin and 45 U/ml of deoxyribonuclease 1. Gradients were prepared by pipetting 2 ml of each density of metrizamide stepwise into 15-ml conical bottom tubes or 7 ml of each density into 50-ml tubes. After the addition of 1.5 ml of the leukocyte suspension, each tube was centrifuged at 1,000 *g* for 45 min at room temperature. The cells at the interface of each layer of metrizamide were collected, diluted with an equal volume of enriched MEM, and analyzed for differential cell types after staining with Wright's and then Giemsa stains. In these experiments, only the cells combined from the 22/23% and 23/24% gradient interfaces and pellets were used. The eosinophils were  $89 \pm 3\%$  pure (mean  $\pm$  SEM, *n* = 15), with neutrophils constituting the remaining contaminating leukocyte. Residual contaminating erythrocytes in eosinophil preparations were eliminated by hypotonic lysis. As assessed by Trypan blue exclusion, cell viability in all fractions was >98%.

*Preparation of Mouse 3T3 Fibroblasts.* The Swiss albino mouse 3T3 fibroblast cell line (American Type Culture Collection, Rockville, MD) was initially seeded into 75-cm<sup>2</sup> tissue culture flasks containing enriched RPMI (RPMI 1640 [Gibco Laboratories] supplemented with 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 10  $\mu$ g/ml gentamycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 10% FCS), and was cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The culture medium was changed every 2–3 d until the fibroblasts reached confluence, which generally took 1 wk. 1 d after confluence was achieved, the fibroblasts were passaged by washing the layers twice with 10 ml of calcium and magnesium-free HBSS (Gibco Laboratories) followed by a 10-min incubation at 37°C with 0.05% trypsin (Gibco Laboratories) in 5 ml of HBSS. After the addition of 20 ml of fresh, enriched RPMI to each flask,  $\sim 5 \times 10^4$  fibroblasts were seeded into 75-cm<sup>2</sup> tissue culture flasks. For coculture experiments,  $\sim 2 \times 10^4$  fibroblasts were seeded

onto 35-mm culture dishes in 2 ml of enriched RPMI; confluence was achieved generally after 7 d, and corresponded to  $0.5\text{--}1.0 \times 10^6$  fibroblasts.

**Culture of Human Eosinophils.** For most experiments, freshly isolated eosinophils at a density of  $0.25\text{--}0.5 \times 10^6$  cells/ml were resuspended in enriched RPMI containing rH GM-CSF<sup>2</sup> and 2 ml of this suspension was seeded into each 35-mm culture dish of confluent 3T3 fibroblasts. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. At 48-h intervals, the culture medium containing the suspension of eosinophils was aspirated, and 1 ml of fresh, enriched RPMI was added directly to each culture dish. The eosinophils in each aspirate were centrifuged at 250 g for 10 min at 4°C, resuspended in 1.0 ml of fresh enriched RPMI 1640 containing rH GM-CSF, and added back to the appropriate original culture dish. For 7-d culture experiments, the medium was not changed on day 6. In some experiments, eosinophils ( $10^6$  cells) were seeded onto fibroblasts, and rH GM-CSF was either not added during the culture or was included only at the time of the initial seeding. In other experiments, eosinophils ( $0.5\text{--}1.0 \times 10^6$  cells) were seeded into culture dishes without 3T3 fibroblasts, and rH GM-CSF (final concentrations, 0.1–100 pM) was added to the initial culture and at each feeding.

**Analysis of the Viability and Density of Eosinophils After Culture.** The eosinophils were recovered from each culture plate by pooling the cells in suspension with those liberated by washing each plate three times with 2 ml of enriched RPMI. The pooled cells were centrifuged at 250 g for 10 min at 4°C and resuspended in 1 ml of enriched MEM. A 50- $\mu$ l sample was added to an equal volume of Trypan blue, and those eosinophils that excluded Trypan blue after a 10-min incubation at room temperature were considered viable. Replicate samples of cells were quantitated using a Neubauer hemacytometer chamber, and were stained with Wright's and then with Giemsa stains. After 7 d of culture all of the leukocytes were similar to freshly isolated peripheral blood eosinophils in that they possessed segmented nuclei and granules that stained reddish-orange; therefore, the survival of the cultured eosinophils was calculated as follows:  $100 \times [(\text{total number of cells}) \times (\text{percent of cells excluding Trypan blue})] / [\text{original number of eosinophils seeded}]$ .

To assess the relative density of the eosinophils after 7 d of culture,  $1\text{--}2 \times 10^6$  cells were suspended in 1.5 ml of enriched MEM, layered over discontinuous gradients of metrizamide, and the initial centrifugation procedure described above was repeated. The cells at each metrizamide interface were collected and diluted with 8 ml of enriched MEM. After centrifugation at 250 g for 10 min at 4°C, the eosinophils were resuspended in 1 ml of enriched MEM and counted. Viability and morphology were assessed as described for fresh eosinophils. When freshly isolated eosinophils ( $10^6$  cells in 1.5 ml) were again layered onto discontinuous metrizamide gradients and centrifuged, they were recovered from the interfaces of normodense fractions.

**Calcium Ionophore-induced Generation of LTC<sub>4</sub>.** Freshly isolated and cultured eosinophils ( $5 \times 10^5$  cells) were suspended in 3 ml of modified Tyrode's buffer, pH 7.5, containing 0.3 mM potassium, 0.1% gelatin, and 20 mM L-serine; the latter amino acid was added to prevent HOCl-dependent oxidative metabolism of LTC<sub>4</sub> (18). After two successive washes with modified Tyrode's buffer at 110 g for 10 min at 4°C, the eosinophils were pooled and resuspended in the same buffer at a density of  $2 \times 10^6$  cells/ml. Samples (100  $\mu$ l) of each cell suspension were prewarmed for 10 min at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and mixed with 100  $\mu$ l of modified Tyrode's buffer with or without 200 pM rH GM-CSF. The eosinophil suspensions were incubated for an additional 60 min at 37°C in a 5% CO<sub>2</sub> atmosphere and activated by incubating the cells for 30 min at 37°C with an additional 200  $\mu$ l of modified Tyrode's buffer containing 5.0  $\mu$ M calcium ionophore A23187. The reaction was terminated by the addition of 2 ml of methanol at 4°C. Each methanolic suspension was placed under argon at 4°C for 14 h, centrifuged at 500 g for 30 min at 4°C to remove proteins and cell debris, and stored at  $-20^\circ\text{C}$  under argon.

To quantitate immunoreactive LTC<sub>4</sub> by RIA, methanolic extracts were evaporated to

<sup>2</sup> rH GM-CSF, expressed in monkey COS cells (26), was purified to homogeneity (27), diluted in PBS containing 0.1% BSA, sterilized by filtration, and stored at 4°C.

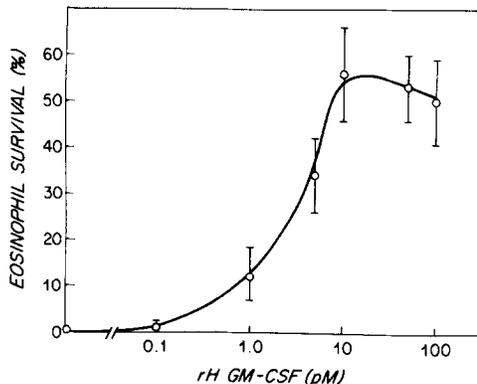


FIGURE 1. Dose-dependent survival of eosinophils cultured for 7 d with incremental concentrations of rH GM-CSF. Each data point is the mean  $\pm$  SEM of three experiments with eosinophils isolated from different donors.

dryness under negative pressure (Speed Vac Concentrator; Savant Instruments, Inc., Hicksville, NY) and resuspended in Tris-Isogel buffer (0.1 M Tris-HCl, 0.14 M NaCl, 0.1% gelatin), pH 7.4. RIA was performed in a 250- $\mu$ l volume of Tris-Isogel buffer containing 100  $\mu$ l of sample, 50  $\mu$ l of  $^3$ H-labeled LTC<sub>4</sub> (~40 Ci/mmol; New England Nuclear, Boston, MA), and 100  $\mu$ l of immune rabbit plasma (18). Unbound  $^3$ H-labeled LTC<sub>4</sub> was absorbed during a 15-min incubation at 4°C with 1 ml of a 10:1 (wt/wt) suspension of charcoal/dextran T-40 in 10% Tris-Isogel buffer. The charcoal was removed by centrifugation at 2,000 g for 15 min at 4°C; the residual radioactivity in the supernatant was quantitated by  $\beta$ -scintillation counting. In this RIA, synthetic LTC<sub>4</sub> was detectable on the linear portion of the radioligand inhibition-binding curves at concentrations of 0.1–1.0 ng.

**Cytotoxicity Assay.** Freshly isolated and cultured eosinophils were washed three times with 1.5 ml of enriched MEM by centrifugation at 110 g for 10 min at 4°C. Cells were resuspended at a density of  $2 \times 10^6$  cells/ml in enriched MEM, and 50- $\mu$ l samples containing  $10^5$  cells were added to each well of a 96-well round-bottomed microtiter plate. 50  $\mu$ l of enriched MEM with or without 200 pM rH GM-CSF was added, and the cells were incubated for 15 min at 37°C in a sealed, humidified box. A 50- $\mu$ l portion of enriched MEM containing pooled, heat-inactivated sera from patients who had been infected with *S. mansoni* (1:100 final dilution) was added to each well along with 50  $\mu$ l of mechanically transformed *S. mansoni* larvae ( $2 \times 10^3$  worms/ml) (14). The viability of the larvae was assessed 24 h later by microscopy using motility, contractility, and toluidine blue exclusion as criteria (19). In some wells, eosinophils were omitted, and it was found that the remaining reagents had no direct effect on the schistosomula.

## Results

**Development of Conditions For Culture of Human Eosinophils with rH GM-CSF in the Presence of 3T3 Fibroblasts.** In initial experiments, when human eosinophils were isolated under sterile conditions and cultured for 7 d at a density of  $10^6$  cells in 2 ml of enriched RPMI alone or enriched RPMI containing 0.1–100 pM concentrations of rH GM-CSF, there was a dose-dependent enhancement of cell survival. Eosinophil viability began to be enhanced at 1 pM rH GM-CSF and reached a plateau at 10 pM rH GM-CSF (Fig. 1). Viability was 50% maximal at ~3 pM rH GM-CSF. When cultured in enriched RPMI alone, no cells survived to day 7. As viewed by light microscopy, eosinophils that were cultured in enriched RPMI containing rH GM-CSF had large well-delineated eosinophilic staining cytoplasmic granules and segmented nuclei.

Because mouse 3T3 fibroblasts have been shown to influence the viability of mast cells (20, 21) and lymphocytes (22) of various species, the 7-d survival of

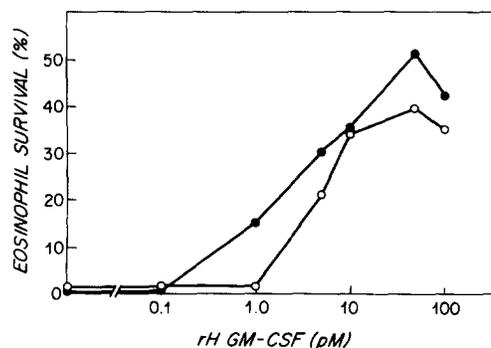


FIGURE 2. Effects of 3T3 fibroblasts on rH GM-CSF-dependent eosinophil viability. Dose-dependent survival of eosinophils from a single donor cultured for 7 d with incremental concentrations of rH GM-CSF in the presence (●) or absence (○) of mouse 3T3 fibroblasts.

TABLE I  
7-d Survival of Eosinophils under Different Culture Conditions

Culture conditions	Survival*	
	%	n
Enriched RPMI 1640 alone	<1	6
Culture with 3T3 fibroblasts alone	1 ± 0	6
rH GM-CSF alone	43 ± 7 <sup>†</sup>	8
Culture with 3T3 fibroblasts and rH GM-CSF	67 ± 6 <sup>†</sup>	8

\* Survival was calculated as described in Materials and Methods. All data are expressed as mean ± SEM.

<sup>†</sup> When eosinophil samples cultured with rH GM-CSF alone were compared with paired samples cultured with rH GM-CSF in the presence of fibroblasts, the increase in survival was significant as determined by the two-tailed Student's *t*-test ( $p < 0.01$ ).

eosinophils in culture was examined with incremental concentrations of rH GM-CSF in the presence or absence of a confluent monolayer of mouse fibroblasts. Eosinophil survival increased modestly at 7 d when eosinophils were cultured with rH GM-CSF in the presence of 3T3 fibroblasts as compared with eosinophils cultured with rH GM-CSF alone, as depicted in the experiment shown in Fig. 2. Few eosinophils (<1%) cultured with fibroblasts alone survived to day 7. In two experiments, the addition of 10–50% fibroblast-conditioned medium to enriched RPMI supplemented with 50 pM rH GM-CSF produced no increase in eosinophil survival at 7 d, as compared with eosinophils cultured in 50 pM rH GM-CSF alone. When the data for survival at 7 d for all culture conditions were tabulated, survival of eosinophils in enriched RPMI alone or with 3T3 fibroblasts alone was ≤1%. When cells were cultured with 50 pM rH GM-CSF alone 43% of the eosinophils survived. A statistically significant further increase in eosinophil survival to 67% occurred when cultured with 50 pM rH GM-CSF in the presence of fibroblasts (Table I,  $p < 0.01$ ). These findings led us to select the combination of 50 pM rH GM-CSF and fibroblasts as the standard culture condition.

When 50 pM rH GM-CSF was present only at the initial seeding of the eosinophils onto the fibroblast layer, an average of 28% of the eosinophils were viable after 7 d in culture ( $n = 2$ ). In contrast, 83% of these cells survived when 50 pM of rH GM-CSF was maintained at each change of media, indicating the need to continually expose the eosinophils to rH GM-CSF during culture with fibroblasts. The time course of eosinophil survival in culture with fibroblasts was

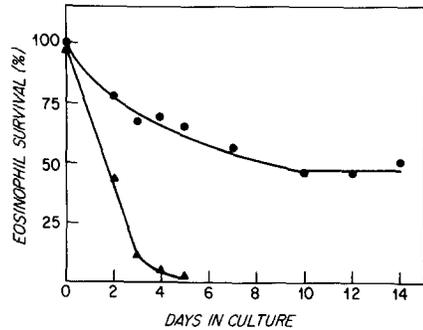


FIGURE 3. Time course of viability of eosinophils in culture. Eosinophils from a single donor were maintained in culture with 3T3 fibroblast alone (▲) or with 3T3 fibroblasts and 50 pM rH GM-CSF (●). Each data point is the average value of duplicate plates.

examined in the presence or absence of 50 pM rH GM-CSF. As is depicted in the representative experiment in Fig. 3, there was a gradual decrease in the percentage of surviving eosinophils through day 10 with no further decline to day 14 for cells cultured with GM-CSF and fibroblasts. The viability of cells cultured with fibroblasts alone declined in a linear fashion such that <10% remained after day 3. After 7 d of culture with 50 pM rH GM-CSF in the presence of fibroblasts in three experiments, including that shown in Fig. 3, eosinophil survival was  $56 \pm 2\%$  (mean  $\pm$  SEM), and at 14 d was  $48 \pm 5\%$  ( $p > 0.2$ ). These results indicate substantial stability for cells that were viable at 7 d. Morphologically, the eosinophils cultured for 7 d with rH GM-CSF in the presence of fibroblasts were uniform in appearance, as illustrated by the representative Wright-Giemsa stain of cytocentrifuged eosinophils after 7 d of culture (Fig. 4). No mitotic figures were seen during culture.

In separate experiments to assess the specificity of this culture system, polymorphonuclear leukocytes were harvested from the 21/22% metrizamide interface consisting of 95% neutrophils and 5% eosinophils and were seeded onto fibroblasts with 50 pM rH GM-CSF. In these two experiments, <2% of the original seeded neutrophils were present after 4 d of coculture, while 100% of the eosinophils were viable. The effect of modest neutrophil contamination upon eosinophil survival was analyzed by comparing the survival of eosinophil preparations of the highest and least purity isolated from comparable metrizamide fractions. When the eosinophils were  $98 \pm 0\%$  pure (mean  $\pm$  SEM,  $n = 4$ ), survival after 7 d of culture with 50 pM rH GM-CSF and fibroblasts was  $58 \pm 7\%$ . Likewise, when eosinophil purity was  $73 \pm 4\%$  ( $n = 3$ ), the viability at 7 d was similar ( $p > 0.50$ ), being  $60 \pm 8\%$ .

*Effect of Culture on the Centrifugation Density of Normodense Eosinophils.* The initial population of eosinophils that was seeded onto 3T3 fibroblasts with 50 pM rH GM-CSF was uniformly normodense, as defined by its equilibration at the 23/24% metrizamide interface and pellet (Fig. 5A). After 7 d of culture with 50 pM rH GM-CSF in the presence of fibroblasts, the eosinophils were washed, resuspended in enriched MEM, layered onto discontinuous metrizamide gradients, and centrifuged. No eosinophils were recovered at the density of the starting cells. Instead, 99% of the cultured eosinophils were harvested from the supernatant/18%, 18/20% (Fig. 5B), and 20/21% metrizamide interfaces, indicating a marked shift to lesser densities and acquisition of the hypodense phenotype. In four experiments, including that depicted in Fig. 5, with eosino-

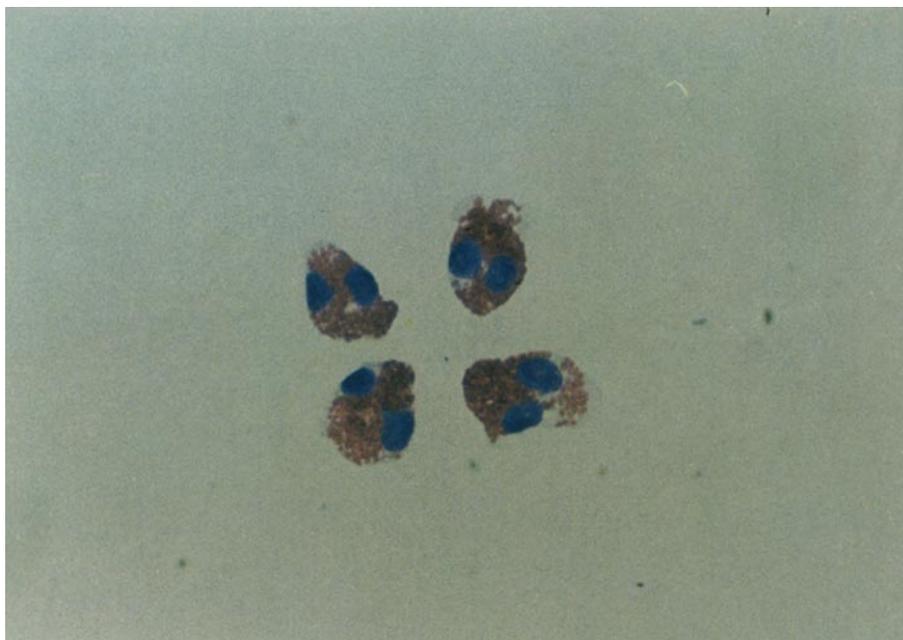


FIGURE 4. Photomicrograph of cultured eosinophils. Normodense eosinophils were cultured with 50 pM rH GM-CSF in the presence of 3T3 fibroblasts for 7 d, and stained with Wright's and Giemsa, respectively.

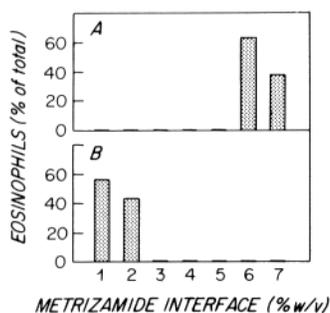


FIGURE 5. The effect of culture on the relative distribution of eosinophils in density gradients. (A) The gradient sedimentation characteristics of the freshly isolated eosinophils. (B) The density gradient profile of the replicates after 7 d of culture. Fractions 1–6 refer to the percent of eosinophils at the medium/18, 18/20, 20/21, 21/22, 22/23, and 23/24% metrizamide interfaces, respectively, and fraction 7 to the combined 24% metrizamide layer and the cell pellet. Recovery of the total number of eosinophils loaded onto the gradient in A was 100% and in B was 82%.

phils isolated from different donors, the eosinophils became uniformly hypodense after 7 d of culture with rH GM-CSF in the presence of fibroblasts (Table II).

*Generation of LTC<sub>4</sub> by Freshly Isolated and by Cultured Eosinophils.* The calcium ionophore-stimulated generation of LTC<sub>4</sub> by eosinophils incubated for 1 h with 50 pM rH GM-CSF was compared with that obtained after 7 d of culture with 50 pM rH GM-CSF in the presence of fibroblasts. In comparison to freshly isolated eosinophils stimulated in buffer alone, acute exposure to rH GM-CSF for 1 h or exposure for 7 d in culture resulted in an ~2.5-fold enhancement of calcium ionophore-stimulated LTC<sub>4</sub> generation (Table III). This degree of enhancement was statistically significant ( $p < 0.05$ ) when compared with freshly isolated eosinophils stimulated in buffer alone. However, no statistical difference in ionophore-stimulated LTC<sub>4</sub> generation ( $p > 0.5$ ) was obtained between those

TABLE II  
Density of Eosinophils before and after Culture

Exp.	Freshly isolated (fraction*)							Cultured (fraction*)						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7
1	0	0	0	0	0	70	30	68	24	6	2	0	0	0
2	0	0	0	0	0	0	100	30	20	45	4	1	0	0
3	0	0	0	0	0	62	38	57	43	0	0	0	0	0
4	0	0	0	0	45	45	10	56	40	4	0	0	0	0
Mean ± SEM	0	0	0	0	11 ±6	44 ±14	45 ±17	53 ±7	32 ±5	14 ±9	1 ±1	0	0	0

\* Fractions 1–6 refer to cells recovered at the medium/18, 18/20, 20/21, 21/22, 22/23, and 23/24% metrizaide interfaces, respectively, and fraction 7 refers to the combined 24% metrizaide layer and the cell pellet. The overall recovery of cultured eosinophils after centrifugation was  $71 \pm 7\%$  (mean  $\pm$  SEM,  $n = 4$ ). The data are expressed as percent of total eosinophils recovered.

TABLE III  
*LTC<sub>4</sub> Generation by Eosinophils before and after Culture*

Exp.	Assay conditions		
	Freshly isolated eosinophils challenged in:		Cultured eosinophils challenged in buffer alone
	Buffer alone	rH GM-CSF added	
1	13	23	37
2	22	93	85
3	25	37	88
4	25	77	52
5	14	21	27
6	43	87	ND
Mean $\pm$ SEM	21 $\pm$ 4	56 $\pm$ 13	58 $\pm$ 12
<i>n</i> value	6	6	5
<i>p</i> value*	—	<0.05	<0.05

Freshly isolated cells were exposed to buffer alone or rH GM-CSF for 1 h before calcium ionophore activation. Eosinophils cultured for 7 d were washed and activated in the absence of added rH GM-CSF. All values are the average of duplicate samples expressed as nanograms/ $10^6$  cells. Eosinophil survival for cells cultured for 7 d with 50 pM rH GM-CSF and 3T3 fibroblasts was  $68 \pm 9\%$  (mean  $\pm$  SEM,  $n = 5$ ).

\* The statistical significance of differences between sample means for each population was based upon comparisons to freshly isolated eosinophils stimulated in buffer alone as determined by the paired two-tailed Student's *t* test.

eosinophil populations exposed for either 1 h or cultured with rH GM-CSF in the presence of fibroblasts.

*Cytotoxicity of Freshly Isolated and of Cultured Eosinophils.* Freshly isolated eosinophils killed  $8 \pm 3\%$  (mean  $\pm$  SEM,  $n = 4$ ) of the *S. mansoni* larvae at 24 h, and the percentage increased to  $51 \pm 9\%$  ( $n = 4$ ) when the assay was performed in the presence of 50 pM rH GM-CSF (Table IV). After 7 d of culture with 50 pM rH GM-CSF in the presence of 3T3 fibroblasts, the resulting eosinophils were washed and their ability to kill *S. mansoni* larvae was assayed. These cultured eosinophils killed  $57 \pm 15\%$  ( $n = 4$ ) of the larvae during the 24-h assay (Table IV). When eosinophils were exposed to rH GM-CSF for 1 h or 7 d, their

TABLE IV  
*Antibody-dependent Cytotoxicity of Eosinophils against S. mansoni before and after Culture*

Exp.	Assay conditions		
	Freshly isolated eosinophils challenged in:		Cultured eosinophils challenged in medium alone
	Medium alone	rH GM-CSF added	
1	2	43	88
2	12	62	48
3	13	70	73
4	3	30	20
Mean $\pm$ SEM*	8 $\pm$ 3	51 $\pm$ 9	57 $\pm$ 15
<i>p</i> value <sup>†</sup>		<0.01	<0.05

Freshly isolated cells were exposed to enriched MEM alone or rH GM-CSF at the time of assay. Eosinophils cultured for 7 d were washed and assayed in medium alone. All values are the average of duplicate or triplicate samples expressed as percent of larvae killed after 24 h. Eosinophil survival for cells cultured for 7 d with 50 pM rH GM-CSF and 3T3 fibroblasts was 68  $\pm$  11% (mean  $\pm$  SEM, *n* = 4). No loss of viability of the larvae occurred in the control wells in which all the reactants except eosinophils were present.

\* *n* = 4.

<sup>†</sup> The statistical significance of differences between sample means for each population was based upon comparison to freshly isolated cells assayed in medium alone as determined by the paired two-tailed Student's *t* test.

antibody-dependent cytotoxicity to helminths was enhanced six- to sevenfold (*p* < 0.05). There was no statistical difference (*p* > 0.5) in the augmented cytotoxicity for eosinophil populations exposed to rH GM-CSF for only 1 h or in culture with rH GM-CSF and fibroblasts for 7 d.

### Discussion

In this study, we describe the biological and biochemical properties of hypodense eosinophils generated in vitro by the culture of normodense peripheral blood eosinophils with rH GM-CSF in the presence of mouse 3T3 fibroblasts. In the presence of 50 pM rH GM-CSF alone, human peripheral blood eosinophils were maintained ex vivo for 1 wk with 43% survival (Table I). Because no corrections were made for cell losses that might have occurred during media changes, the percent survival may have been underestimated. The dose-response characteristics of the eosinophils in this assay of survival (Fig. 1) were nearly identical to those described for the acute GM-CSF-dependent enhancement of human eosinophil-mediated, antibody-dependent cytotoxicity and calcium ionophore-stimulated leukotriene synthesis (14). This effect of rH GM-CSF on eosinophil viability occurs in a range of concentrations that could be mediated by a high-affinity GM-CSF binding site, such as that of human neutrophils, which has an apparent  $K_d$  of 20 pM (13).

Because mouse 3T3 fibroblasts have been shown to influence the viability of mast cells and lymphocytes of various species (20–22), the ability of these cells to improve the viability of eosinophils cultured with rH GM-CSF was examined. Although fibroblasts alone did not maintain eosinophil viability, the mean survival of eosinophils cultured with confluent fibroblasts and rH GM-CSF improved

to 67% at 7 d (Table I) and this level of viability was maintained at 14 d (Fig. 3). As assessed by light microscopy, the cultured cells appeared similar to the initially seeded eosinophils (Fig. 4). The addition of fibroblast-conditioned medium to enriched RPMI supplemented with 50 pM GM-CSF had no beneficial effect. This suggests that if a soluble fibroblast factor was responsible for the enhanced survival, it was short lived. A detoxification role for 3T3 fibroblasts has been proposed for the growth of B cell clones (22); similarly, fibroblasts may act as scavengers during eosinophil culture, removing cytotoxic granular proteins released when the eosinophils are manipulated. Enhanced eosinophil survival in culture was dependent upon the continued addition of fresh rH GM-CSF to the culture system during each change of media, as demonstrated by the decreased survival of eosinophils that were exposed only once to rH GM-CSF at the initial seeding.

In earlier studies (23, 24) with femtomolar doses of rH GM-CSF, the survival of both human eosinophils and neutrophils increased only 6–9 h. In the present study, the remarkably prolonged survival of eosinophils to 14 d obtained with GM-CSF and 3T3 fibroblasts is probably a consequence of using concentrations of rH GM-CSF that are appropriate to the apparent  $K_d$  of its receptor on other cells, and culturing the eosinophils in dishes that permit the media to be changed frequently and replenished with fresh media containing rH GM-CSF. When neutrophils were cultured for 4 d, either as a contaminating cell type or as the major population of leukocytes, only viable eosinophils were recovered, indicating that in this culture system with mouse 3T3 fibroblasts, rH GM-CSF preferentially enhances the survival of eosinophils.

The eosinophils used in this study were specifically selected for their normal density, as distinct from the less dense eosinophils of most hypereosinophilic donors. Whereas the cells were initially isolated from the metrizamide density interfaces >22/23%, after culture they were recovered in the supernatant/18%, 18/20%, and 20/21% metrizamide interfaces (Fig. 5 and Table II), indicating a change to hypodense. These hypodense eosinophils exhibited enhanced calcium ionophore-stimulated generation of LTC<sub>4</sub> and enhanced antibody-dependent cytotoxicity to *S. mansoni* larvae. The levels of LTC<sub>4</sub> that were generated by eosinophils that survived 7 d of culture with rH GM-CSF in the presence of fibroblasts were comparable to those obtained by incubation of cells with rH GM-CSF for only 1 h (Table III). This finding indicates that the 2.5-fold augmentation of this function achieved during short-term exposure to GM-CSF is not diminished with prolonged exposure of the cell to the cytokine. Exposure to rH GM-CSF acutely augmented the ability of eosinophils to mediate antibody-dependent cytotoxicity for *S. mansoni* by approximately sevenfold, and this also did not diminish over 7 d of culture (Table IV). Both enhanced ionophore-stimulated LTC<sub>4</sub> generation and helminthic cytotoxicity have been ascribed to hypodense eosinophils freshly isolated from hypereosinophilic donors (15, 16). The observation that normodense eosinophils cultured with GM-CSF in the presence of 3T3 fibroblasts can be transformed to hypodense cells with an augmented capacity to elaborate LTC<sub>4</sub> and to mediate antibody-dependent cytotoxicity suggests that this phenotypic change may be regulated in vivo by GM-CSF.

The demonstration that rH GM-CSF both prolongs the survival of human eosinophils and is associated with enhancement of their biochemical and cytotoxic capacities is further evidence of the ability of specific human colony-stimulating factors to exhibit activity for normal mature peripheral blood polymorphonuclear leukocytes. In addition, it suggests that a cytokine released locally in the tissues might act to prolong the life span of recruited eosinophils *in vivo*. The augmented biological activities and the enhanced survival of eosinophils exposed to GM-CSF would be of obvious benefit to the host at those tissue sites where helminths have penetrated (25).

### Summary

Normodense human peripheral blood eosinophils were isolated under sterile conditions from the 22/23 and 23/24% interfaces and the cell pellet of metrizamide gradients. After culture for 7 d in RPMI media in the presence of 50 pM biosynthetic (recombinant) human granulocyte/macrophage colony-stimulating factor (rH GM-CSF),  $43 \pm 7\%$  (mean  $\pm$  SEM,  $n = 8$ ) of the cells were viable; in the absence of rH GM-CSF, no eosinophils survived. The rH GM-CSF-mediated viability was concentration dependent; increased survival began at a concentration of 1 pM, a 50% maximal response was attained at  $\sim 3$  pM, and a maximal effect was reached at concentrations of  $\geq 10$  pM rH GM-CSF. In the presence of rH GM-CSF and mouse 3T3 fibroblasts,  $67 \pm 6\%$  (mean  $\pm$  SEM,  $n = 8$ ) of the eosinophils survived for 7 d. In a comparative analysis, there was no difference in eosinophil viability after 7 and 14 d ( $n = 3$ ) in the presence of 50 pM GM-CSF and fibroblasts. Culture with fibroblasts alone did not support eosinophil survival. The addition of fibroblast-conditioned media to rH GM-CSF did not further improve eosinophil viability, indicating a primary role for GM-CSF in supporting these eosinophil cell suspensions *ex vivo* and a supplementary role for 3T3 fibroblasts. Eosinophils cultured for 7 d localized on density gradient sedimentation at the medium/18, 18/20, and 20/21 interfaces of metrizamide gradients, indicating a change to the hypodense phenotype from their original normodense condition. In addition, the cultured eosinophils generated  $\sim 2.5$ -fold more LTC<sub>4</sub> than freshly isolated cells when stimulated with the calcium ionophore A23187 and manifested sevenfold greater antibody-dependent killing of *S. mansoni* larvae than the freshly isolated, normodense cells from the same donor. Thus we demonstrate the rH GM-CSF dependent conversion *in vitro* of normodense human eosinophils to hypodense cells possessing the augmented biochemical and biological properties characteristic of the hypodense eosinophils associated with a variety of hypereosinophilic syndromes. In addition, these studies provide a culture model of at least 14 d suitable for the further characterization of hypodense eosinophils.

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