Differentiation In Vitro of Hybrid Eosinophil/Basophil Granulocytes: Autocrine Function of an Eosinophil Developmental Intermediate

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Summary

Granulocytes with the hybrid characteristics of eosinophils and basophils have been identified in the bone marrow and peripheral blood of humans with myeloid leukemias. We now describe a technique by which such hybrid granulocytes can be developed in vitro from normal cord blood precursors cultured in the presence of recombinant human interleukin (rhIL) 3 (350 pM) and rhIL-5 (200 pM) in a plastic vessel coated with Matrigel™. After 14 d in culture, 90 ± 3% (mean ± standard error of the mean) of the nonadherent cells cultured in the Matrigel-coated flasks contained both eosinophil and basophil granules, as indicated by staining with Wright's and Giemsa stains. Of the nonadherent cells, 93 ± 1% contained cyanide-resistant peroxidase, and 88 ± 2% were toluidine blue–positive, characteristic of eosinophil and basophil granules, respectively. Transmission electron micrographs showed hybrid cells containing ultrastructurally distinct eosinophil granules with developing crystalline cores and basophil granules with reticular structures. These 14-d cord blood–derived cell cultures showed strong hybridization signals for eosinophil-derived neurotoxin by RNA blot analysis and contained 78 ng histamine per 10^6 cells. When the granulocytes were removed from cytokine-containing medium and suspended without Matrigel in RPMI 1640 medium containing 10% fetal calf serum (FCS), more than 80% of the granulocytes excluded trypan blue for as long as 5 d, and 93% had developed into eosinophils at 6 d. Conditioned medium prepared over 48 h from the 14-d cell cultures (hybrid granulocytes) sustained the 4-d viability in vitro of 78% of peripheral blood eosinophils from atopic donors. In comparison, 13% survived in RPMI 1640 containing 10% FCS alone. This viability-sustaining activity was nearly completely neutralized by an anti–granulocyte/macrophage colony-stimulating factor (GM-CSF) antibody and was only minimally reduced by anti–IL-3 or –IL-5. Thus, cells possessing both eosinophil and basophil granules by both histochemical and ultrastructural analysis can be developed from normal progenitors in vitro in response to eosinophilopoietic cytokines and Matrigel. Their subsequent spontaneous development into mature eosinophils suggests that hybrid granulocytes are part of a normal developmental sequence during eosinophilopoiesis. Furthermore, these hybrid granulocytes are capable of autoregulation through elaboration of GM-CSF, which sustains their viability.

Eosinophils and basophils have many common characteristics, such as granules that contain major basic protein (1) and lysophospholipase (2), a 5-lipoxygenase pathway (3–5), and receptors for IL-3, IL-5, and GM-CSF (6, 7). Patients with basophilia often have eosinophilia (8), and a rare syndrome has been described characterized solely by the absence of both cell types (9). Thus, although differences exist between mature basophils and eosinophils, such as the presence of histamine in basophil granules (10), granule ultrastructure (11), and staining properties of the granules and nuclear morphology (8), evidence supports a common lineage.

It has been suggested that eosinophils and basophils arise from a common eosinophil/basophil lineage intermediate that has coexistent eosinophil and basophil granules ("hybrid" granulocytes) (10, 12). Such hybrid granulocytes have been identified in the peripheral blood of patients with chronic myelogenous leukemia (12, 13) and in the marrow of patients with M4 type acute myelogenous leukemia (14, 15). Furthermore, when peripheral blood leukocytes from M4-type acute myelogenous leukemia patients were transplanted into mice with severe combined immunodeficiency that were then treated with intraperitoneal c-*kit* ligand and a fusion cytokine

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containing both IL-3 and GM-CSF, leukocyte engraftment resulted and cells that resembled eosinophils with prominent basophilic granules appeared in both the marrow and the peripheral circulation (16). Based on the criteria of cell size and nuclear morphology, hybrid granulocytes have been characterized as abnormal eosinophils having basophil granules (14–16) or basophils having eosinophil granules (12, 13). These cells exhibit metachromatic staining with toluidine blue and contain a cyanide-resistant peroxidase characteristic of eosinophils (12). Additional experimental support for a hybrid eosinophil/basophil progenitor includes the observation of leukocytes with both eosinophil and basophil granules in hematopoietic colonies derived from normal peripheral blood progenitors grown in semisolid methylcellulose medium supplemented with conditioned medium from a hairy cell leukemia cell line, Mo (10). These findings suggest that such "ambiguous" cells might be part of a normal developmental sequence that is more readily apparent in patients with certain hematologic malignancies.

Various techniques have been described for the development of eosinophils and basophils in vitro from peripheral blood, cord blood, and bone marrow progenitors (10, 11, 17–19). When cord blood–derived precursors were cultured in the presence of recombinant human (rh)[1] IL-3, the cell population contained 39% eosinophils and 22% basophils after 14 d and 81% eosinophils after 21 d (17). Cord blood progenitors maintained in culture for 21–35 d in the presence of both rhIL-3 and rhIL-5 developed into populations of 80–95% eosinophils, with basophils among the remaining cells (20). Only the study of Denburg et al. (10), which used an uncharacterized conditioned medium to develop eosinophil and basophil colonies, found that 5–48% of the cells possessed both granule types.

We now describe the development of ~90% pure eosinophil/basophil hybrid granulocytes from human umbilical cord blood mononuclear cells cultured with the cytokines rhIL-3 and rhIL-5 along with a soluble extracellular matrix (Matrigel™). The inclusion of the latter appears to accelerate the acquisition of granules and supports the development of the hybrid phenotype. It appears likely that this developmental intermediate is overrepresented in certain myelogenous leukemias (12–16). The fact that these hybrid granulocytes express a third eosinophilopoietic cytokine distinct from those used for their development represents a previously unrecognized autoregulatory capability in this lineage.

Materials and Methods

Culture System for Generating Granulocytes

Cord blood was drawn from the umbilical vein of the placenta after elective cesarean section deliveries under sterile conditions into 50-ml syringes containing 1,000 U heparin. The blood was layered onto a cushion of Ficoll-Paque™ (1.77 g/ml) in 50-ml sterile conical-bottom centrifuge tubes (1:1 vol/vol blood/Ficoll). After centrifugation of the tubes at 350 *g* for 30 min at room temperature, the mononuclear leukocytes at the interface were collected and pelleted (17). Contaminating erythrocytes were removed by hypotonic lysis with sterile 0.2% NaCl. The cells were counted in a hemocytometer, and cytospin slides were prepared and stained with Wright's and Giemsa stains to provide an initial differential count. The mononuclear cells were suspended in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) containing 10% FCS, 50 μM β-mercaptoethanol, 200 pM rhIL-5 (R & D Systems, Minneapolis, MN), and 350 pM rhIL-3 (R & D Systems) at a density of 0.5–1.0 × 10^6 cells per ml, and they were transferred with a pipette into culture flasks coated with 0.5 ml/cm^2 Matrigel (Collaborative Research, Inc., Bedford, MA) (21–24). As a control for the effects of Matrigel, duplicate cells were suspended in an uncoated flask. The culture medium containing the cytokines was changed every 7 d, at which time cell counts were obtained and cell viability was assessed by the exclusion of trypan blue.

Histochemical, Ultrastructural, and Biochemical Analyses of Cord Blood–derived Granulocytes

Differential Cell Counts. On defined days of culture, 10^4 cells were applied to glass slides by cytocentrifugation. The slides were air dried for 10 min and then immersed in Wright's stain for 2 min. A drop of Giemsa stain was added to each slide and allowed to diffuse into the cells for an additional 2 min. The slides were then rinsed sequentially with distilled water, 70% ethanol, and distilled water, air dried, and mounted with undiluted Permount™ (Fisher Scientific). Differential cell counts were obtained by light microscopy. A cell was classified as either an eosinophil or a basophil if at least three granules of that single cell type were present and no granules of the other cell type were visible. A cell was classified as hybrid if it contained at least one eosinophilic and one basophilic granule. Eosinophils, basophils, and cells with both granule types were expressed as percentages of a total of 300 cells counted.

Cyanide-resistant Peroxidase. Cytospin slides were prepared as above, air dried, fixed in 4% picric acid at room temperature for 30 min, and rinsed in water at room temperature to remove excess fixative. The fixed slides were incubated at room temperature for 10 s in the presence of cyanide buffer (4.9 mg potassium cyanide in 10 ml PBS, pH 6.0), washed in water at room temperature, and pre-incubated for 10 min at room temperature in 10 ml 0.05 M Tris buffer, pH 7.6, containing 5 mg diaminobenzene and 5% sucrose. Then, 0.1 ml of fresh 1% H_2O_2 was added to the buffer, and the slides were incubated in this mixture for 1 h at room temperature. After incubation, the slides were washed three times in the 0.05 M Tris buffer, air dried, and mounted with Immuno-mount (Shandon, Pittsburgh, PA). Cells having dark brown staining characteristic of the eosinophil-specific cyanide-resistant peroxidase (12) were expressed as the percentage positive of 300 cells inspected visually on each cytocentrifugation slide.

Toluidine Blue Metachromasia. Cytospin slides were prepared and air dried as described above. The slides were then immersed in toluidine blue, pH 6.6, for 10 min, rinsed in distilled water, air-dried for 30 min, and mounted in Permount. 300 cells were counted on each slide, and the proportion of cells exhibiting metachromatic staining indicative of basophilic granules was expressed as a percentage of the total.

Granule Ultrastructure. Cell pellets (5–10 × 10^6) of cord blood–derived granulocytes cultured for 14 d in rhIL-5 and rhIL-3 in the presence of Matrigel were fixed in 3% glutaraldehyde, 2% paraformaldehyde, 0.1% sodium cacodylate buffer, pH 7.4, for 2 h at room temperature. The fixed cells were washed three times for 5 min each in 0.1 M sodium cacodylate buffer with 5% sucrose, pH 7.4.

[1] *Abbreviations used in this paper:* EDN, eosinophil-derived neurotoxin; rh, recombinant human.

Samples were postfixed with 1% OsO_4 in 0.1 M acetate veronal buffer, pH 7.4, for 1 h at 4°C and enhanced with 0.5% uranyl acetate in 0.1% acetate veronal buffer, pH 6.0, for 1 h at 37°C in the dark. The pellets were dehydrated and embedded in Epon according to standard procedures (25). Grey to silver sections were cut using a Reichert-Jung Ultracut Microtome (Leicha, Inc., Deerfield, IL), stained with uranyl and lead salts, and examined in a microscope (100 CX; JEOL Ltd., Tokyo, Japan) operating at 80 KV. All cells were assessed for eosinophil, basophil, or hybrid granule populations.

Histamine Content. The histamine content of the granulocytes was assessed by a radioimmunoassay (Amacs, Inc., Westbrook, ME). After 14 d in culture, the cells were suspended in sterile saline at a concentration of 10^6 cells per ml and subjected to three freeze-thaw cycles. The resultant slurry was diluted 1:10, 1:100, and 1:1,000 before ELISA analysis. This assay system is capable of detecting as little as 0.05 ng histamine (10). Results were expressed as nanograms of histamine per 10^6 cells.

Steady-state Transcription of Eosinophil-derived Neurotoxin (EDN). To further characterize the in vitro–derived granulocytes according to their biosynthesis of a mRNA species encoding an eosinophil granule-specific protein, RNA blot analysis was performed on RNA samples extracted from cord blood mononuclear cells that had been cultured for 3, 7, 10, and 14 d with rhIL-3 and rhIL-5, in both the presence and the absence of Matrigel. Total RNA was extracted with TRI Reagent™ (Molecular Research, Inc., Cincinnati, OH) from 10^7 cells pelleted by centrifugation. A 5–10-μg sample of total RNA was loaded into the wells of a 1.3% agarose gel containing 1× 3-[*N*-morpholino]propane-sulfonic acid (MOPS) buffer, pH 7.0, and 20% formaldehyde. After electrophoresis, RNA was transferred from the gel to Magnagraph™ nylon membranes (Micron Separation, Inc., Westboro, MA) overnight by capillary action. The resultant blot was baked at 80°C for 1 h, then prehybridized in 5× SSC, 2× Denhardt's solution, 0.25% SDS, 100 μg/ml salmon sperm DNA, and 50% formamide overnight in a 43°C Hybaid oven (National Labnet Co., Woodbridge, NJ). A full-length cDNA encoding human EDN was generated by reverse transcriptase–PCR. Briefly, total RNA from a preparation of 98% pure peripheral blood eosinophils (see below) was extracted with the TRI Reagent. Reverse transcription was performed after a 5-μg portion of total RNA was primed with oligo dT with a commercial kit (cDNA Cycle Kit; Invitrogen, San Diego, CA). The resultant DNA preparation was used in a PCR reaction with a sense primer identical to nucleotides 1–30 of the human EDN and an antisense primer to the complimentary strand of nucleotides 680–709 (26). PCR reactions were carried out for 30 cycles of amplification, with a denaturating step at 94°C for 45 s, an annealing step at 60° for 45 s, and an extension step at 72° for 1 min. The resultant PCR product of ~700 bp was resolved on an ethidium bromide–stained 1% agarose gel. The product was isolated and extracted from 1% low melt agarose by phenol/chloroform and then chloroform extraction followed by ethanol precipitation. The specificity of the probe was confirmed according to the characteristic pattern of restriction digests with PstI (Pharmacia Biotech Inc., Piscataway, NJ) and was based on the expected 0.7-kb hybridization signal obtained on RNA blot analysis. This DNA was randomly labeled with [^{32}P]dCTP (New England Nuclear, Boston, MA) using a commercial kit (Prime-it 2 Kit; Stratagene, La Jolla, CA), and was used to probe the RNA blot. After overnight probing, the membrane was washed in 2× SSC, 0.1% SDS at room temperature for 30 min, then in 0.2× SSC at 55°C for 15 min. Hybridization signals were obtained by autoradiography for 1 h onto Kodak XAR film.

Analysis of the Role of Hematopoietic Cytokines in Maintaining the Viability of 14-d Cord Blood–derived Granulocytes

Determination of Cell Viability during Culture after Cytokine Removal. To assess the dependence of 14-d in vitro–derived granulocytes on exogenous hematopoietins for their survival in vitro, one set of cell cultures was continued with various doses of rhIL-5 alone, and replicate samples were maintained in the absence of any exogenous cytokines. The 14-d cord blood–derived granulocytes were washed three times in sterile cold PBS containing 10% FCS, suspended at a concentration of 10^6 cells per ml in RPMI 1640 with 10% FCS and no exogenous cytokines, and pipetted into 96-well microtiter dishes in the absence or presence of incremental concentrations of rhIL-5. The viability of the cells was expressed as a percentage of 100 cells excluding trypan blue dye. These assays were extended for 5 d without additional medium or cytokines.

Preparation of Conditioned Medium from Hybrid Granulocytes and Characterization of its Bioactivity

To determine if the granulocytes derived in vitro were elaborating a soluble viability-sustaining activity, their conditioned medium was assessed for its cytoprotective activity for normal peripheral blood eosinophils. After cord blood–derived mononuclear cells were cultured for 14 d with rhIL-5 and rhIL-3 on Matrigel, 2 × 10^6 cells per ml were washed extensively into RPMI containing 10% FCS alone. The cells were plated on plastic dishes for 48 h, and the resultant supernatant was collected and filtered through a 0.2-μm filter. To assess this conditioned medium for viability-sustaining activity(s), human peripheral blood eosinophils were isolated by an immunomagnetic separation technique (27). Briefly, whole blood was drawn from atopic donors into 50-ml syringes containing 1,000 U sterile heparin, and the cells were sedimented with dextran at 37°C for 1 h. The granulocytes were collected by centrifugation through Ficoll-Paque (1.77 g/ml) at 350 *g* for 30 min at 4°C. After the red blood cells were removed by repeated hypotonic lysis, the granulocyte pellets were incubated for 40 min at 4°C with immunomagnetic beads coupled to a rabbit anti–human CD16 antibody (Miltenyi, Sunnyvale, CA). The granulocytes were then suspended in 25 ml PBS at 4°C and eluted through a magnetic column (Miltenyi). The nonadherent eosinophil fraction was quantitated by counting in a hemocytometer. To assess purity, differential cell counts were performed after cytocentrifugation and staining with Wright's and Giemsa stains. Eosinophils were suspended at 10^6 cells per ml in RPMI 1640 with 10% FCS alone, in RPMI 1640 supplemented with 10 pM rhIL-5, or in RPMI 1640 enriched with 50% conditioned medium (28, 29).

The identity of the eosinophil viability-sustaining activity found in the conditioned medium was sought with neutralization bioassays using monospecific polyclonal rabbit antibodies directed against human IL-3, IL-5, and GM-CSF (Genzyme Corp., Cambridge, MA). A saturating concentration of antibody was added to a portion of the wells in 96-well microtiter plates containing RPMI 1640 supplemented with 10% FCS and 25% of the 14-d in vitro–derived granulocyte-conditioned medium. After a 2-h preincubation, 10^5 peripheral blood eosinophils were added to each well. Concentrations of antibody were chosen based upon a dose–response in which the optimal concentrations for the neutralization of 10 pM rhIL-3, 10 pM rhIL-5, and 10 pM rhGM-CSF were determined. For anti–IL-3 and anti–IL-5, optimal neutralization was achieved at concentrations of 100 μg/ml; for anti–GM-CSF, a concentration of 50 μg/ml was optimal. Cell viability was assessed by exclusion of trypan blue by 200 cells after 96 h in culture.

Statistical Analysis. The statistical significance of differences be-

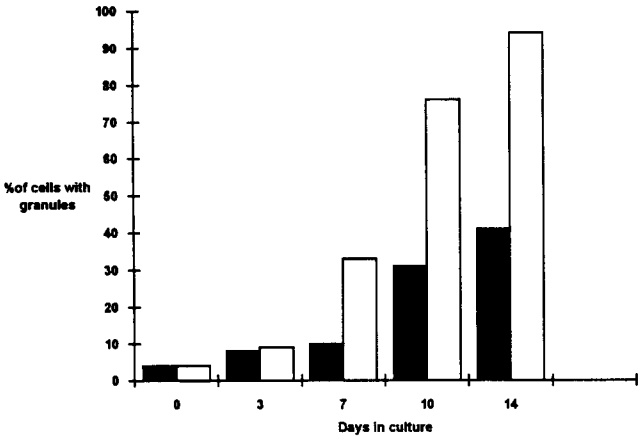

Figure 1. Eosinophil/basophil granule development over 14 d as assessed by staining with Wright's and Giemsa stains of cells derived in vitro from cord blood mononuclear cells cultured in the presence of rhIL-3 and rhIL-5 with (*white bars*) and without (*black bars*) a thin layer of Matrigel. A cell was registered as granulated if it contained three or more eosinophilic or basophilic granules (n = 6, SEM noted in text).

tween sample means for each set of cells was based on comparison as determined by the two-tailed Student's t test. Results are expressed as mean ± SEM.

Results

Histochemical, Biochemical, and Ultrastructural Analyses of Cord Blood–derived Granulocytes

Wright's and Giemsa Staining. After the cord blood mononuclear cells were cultured for 3 d with rhIL-3 and rhIL-5 in the presence or absence of Matrigel, <10% of the cells grown under either culture condition possessed eosinophil or basophil granules (Fig. 1). Over the next 11 d of culture, the cells developed in the presence of Matrigel acquired cytoplasmic granules in a far greater proportion than the cells developed in its absence. At 14 d of culture, 94 ± 3% (n = 6) of the cells grown on Matrigel had developed granules identifiable as basophilic (blue granules) or eosinophilic (red granules) by staining with Wright's and Giemsa stains; 90 ± 3% of the Matrigel-cultured cells contained both eosinophilic and basophilic granules (Fig. 2 *A*), and the remaining 4% with granules had either eosinophil or basophil granules only. In contrast, only 41 ± 3% of the cells grown on plastic had cytoplasmic granules (P <0.01 compared to replicate cells grown on Matrigel) (Fig. 1); 5 ± 3% of the total cells developed in the absence of Matrigel had both eosinophil and basophil granules, 25 ± 2% had eosinophil granules only, and 11 ± 2% had basophil granules only. The remaining cells grown under either culture condition after 14 d were predominantly mononuclear cells lacking visible granules.

Assessment for Metachromasia and Cyanide-resistant Peroxidase. To further characterize the granules of the cells possessing both eosinophil and basophil granules, these cells were stained with toluidine blue and were assessed for cyanide-resistant peroxidase after 14 d of culture. The hybrid nature of the granulation was supported by the findings that with toluidine blue staining, 88 ± 2% of the nonadherent cells were metachromatic (having basophil-like granules) (Fig. 2 *B*) (n = 4), and 93 ± 1% stained intensely for cyanide-resistant peroxidase (having eosinophil-like granules) (Fig. 2 *C*) (n = 3). In contrast, when replicate human umbilical cord blood cells were cultured for 14 d with rhIL-3 and rhIL-5 without Matrigel, 16 ± 2% of the nonadherent cells were metachromatic after staining with toluidine blue (n = 3, P <0.05 compared to replicate cells grown on Matrigel), and 37 ± 9% were positive for cyanide-resistant peroxidase (n = 2; average ± range).

Biochemical Assessment of Hybrid Granulocytes. To assess whether granulocytes exhibiting both eosinophilic and basophilic granules had other specific markers for both eosinophils and basophils, the transcription of EDN (eosinophil marker) and histamine content (basophil marker) were measured. Hy-

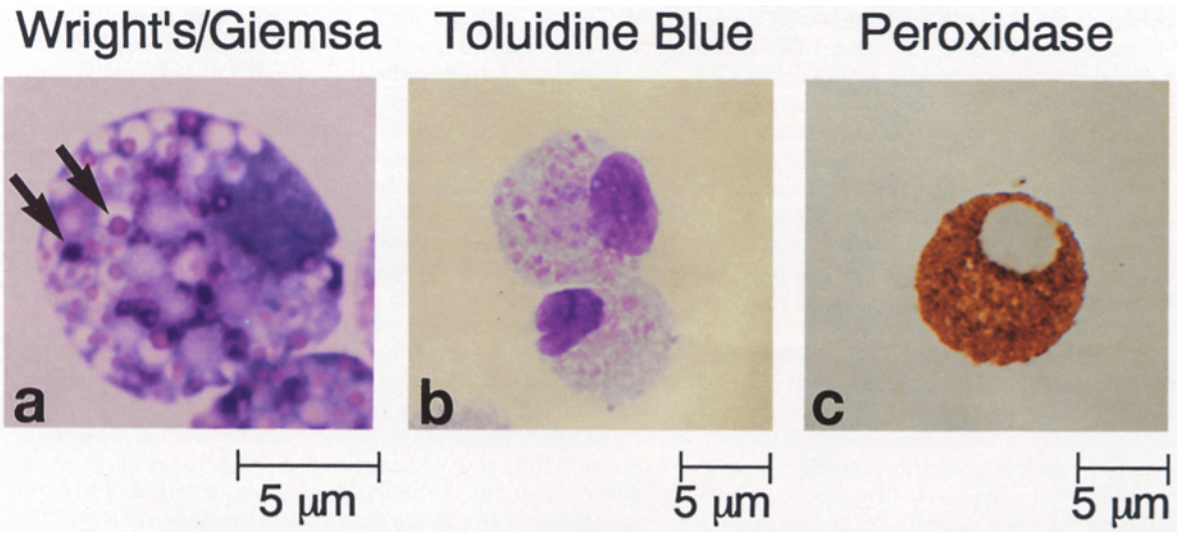

Figure 2. 14-d cord blood–derived hybrid granulocytes stained (*A*) with Wright's and Giemsa stains showing individual eosinophil (*right arrow*) and basophil (*left arrow*) granules (×100), (*B*) with toluidine blue (×60), and (*C*) for cyanide-resistant peroxidase (×60).

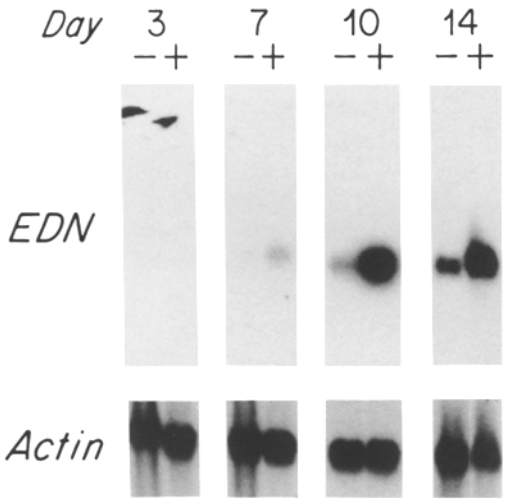

Figure 3. RNA blot analysis for EDN and β-actin of cord blood–derived cells cultured over a 14-d period in the presence (+) or absence (−) of Matrigel.

bridization signals for EDN were strong at 10 and 14 d of culture and were consistently greater for cells developed in Matrigel-coated flasks than in uncoated flasks (Fig. 3). By 14 d in culture, 96% of the population that was developed on Matrigel and used to prepare the RNA had both eosinophil and basophil granules, while only 8% of the cells developed without Matrigel had both granule types.

By radioimmunoassay, preparations of cells maintained in medium supplemented with rhIL-3 and rhIL-5 in the presence of Matrigel contained 78 ± 16 ng histamine/10^6 cells (n = 6). By staining with Wright's and Giemsa stains, these preparations contained 81 ± 2% hybrid granulocytes and 1 ± 0.3% basophils. No mast cells were present. In contrast, 5 × 10^6 freshly isolated peripheral blood eosinophils of 98% purity did not contain measurable histamine.

Ultrastructural Analysis of Hybrid Granulocytes. For transmission electron microscopy, cells developed from cord blood cells by culture for 14 d in medium supplemented by rhIL-3 and rhIL-5 on a bed of Matrigel were examined. More than 80% of the cells were hybrids by Wright's and Giemsa staining. 8 of the 10 cells examined had reticular structures consistent with basophil granules and maturing crystalloid cores consistent with eosinophil granules (11) (Fig. 4).

Analysis of the Role of Hematopoietic Cytokines in Maintaining the Viability of 14-d Cord Blood–derived Granulocytes

Cell Viability during Culture after Cytokine Removal. To assess whether eosinophil hematopoietins or Matrigel was responsible for maintaining a cell population with mixed granules, the staining characteristics of these cells cultured in the absence of cytokines and extracellular matrix proteins were compared with those of a replicate population maintained under the original culture conditions. Cells developed in vitro from cord blood progenitors cultured for 14 d with rhIL-3 and rhIL-5 on Matrigel were resuspended in RPMI 1640 with 10% FCS only and cultured for an additional 6 d in a plastic vessel without Matrigel. After 6 d in culture (total of 20 d), 93 ± 2% of these cells contained eosinophil granules only, 3 ± 2% contained basophil granules only, none were hybrids, and 4 ± 3% were mononuclear cells lacking visible granules (Fig. 5 *C*) (n = 2; average ± range). In contrast, of the cells maintained for an additional 6 d in RPMI 1640 supplemented with rhIL-3 and rhIL-5 on a layer of Matrigel, 68 ± 0% were hybrids exhibiting both eosinophil and basophil granules (Fig. 5 *B*). An additional 27 ± 2% had eosinophil granules only, 2 ± 1% had basophil granules only, and 3 ± 2% lacked visible granules (n = 2, average ± range).

The survival of the 14-d hybrid granulocytes in the absence of exogenous cytokines was remarkably extended in comparison to the survival of freshly isolated eosinophils from

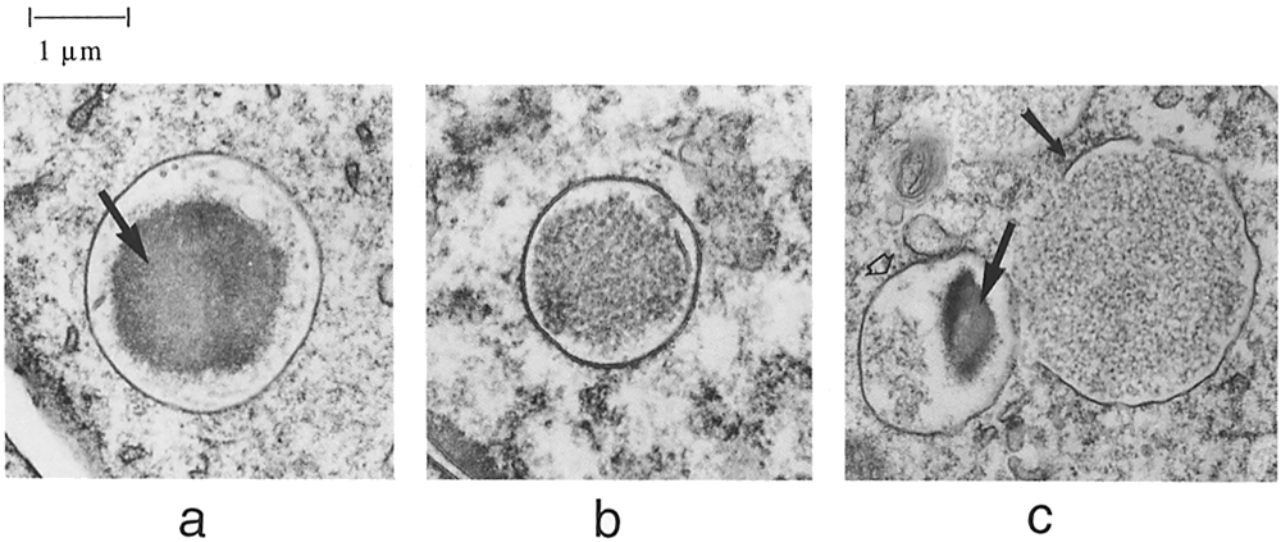

Figure 4. Transmission electron micrograph showing granules of cord blood–derived granulocytes cultured for 14 d in rhIL-5 and rhIL-3 with Matrigel. (*A* and *B*) Individual immature eosinophil granule with a developing core (*arrow*) and a basophil granule, respectively. (*C*) Adjacent eosinophil (*lower arrow*) and basophil (*upper arrow*) granules.

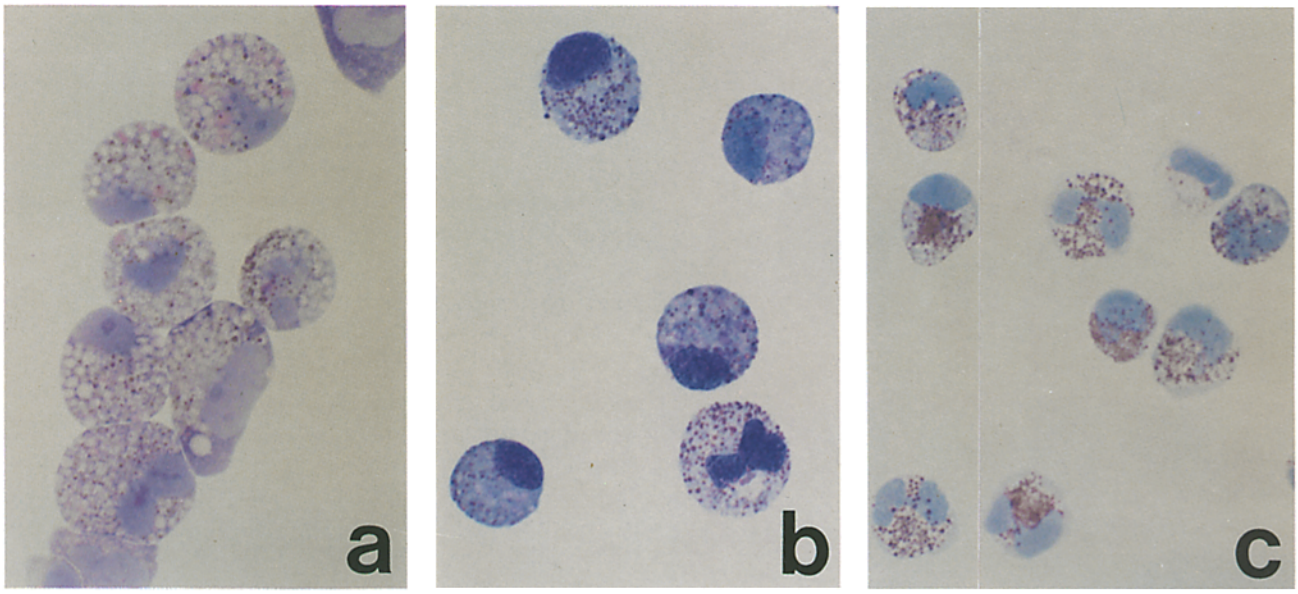

Figure 5. Staining with Wright's and Giemsa stains of cord blood–derived granulocytes showing their hybrid nature at 14 d of culture (*A*) and their differentiation with an additional 6 d of culture in the presence (*B*) or absence (*C*) of rhIL-3, rhIL-5, and Matrigel.

peripheral blood of normal donors (n = 3) (Fig. 6). The survival of the 14-d granulocytes in the absence of exogenous rhIL-5 was 84 ± 6% at 5 d and was not significantly different from replicates cultured with incremental doses of rhIL-5. In contrast, <10% of freshly isolated peripheral blood eosinophils survived for 96 h under the same culture conditions.

Elaboration of Eosinophilopoietic Cytokine(s) by Hybrid Granulocytes. After washing the 14-d hybrid granulocytes, their conditioned medium was harvested after 48 h of culture in RPMI 1640 with 10% FCS alone. An average of 78 ± 8% of freshly isolated peripheral blood eosinophils survived for 96 h when cultured in RPMI 1640 supplemented with 25% conditioned medium (n = 3). 13% of these freshly isolated peripheral blood eosinophils were viable in RPMI 1640 with FCS alone, whereas 93 ± 2% were viable in the presence of 10 pM rhIL-5.

To seek the identity of the hybrid granulocyte-derived viability-sustaining activity, neutralization bioassays were used with polyclonal monospecific antibodies directed against the

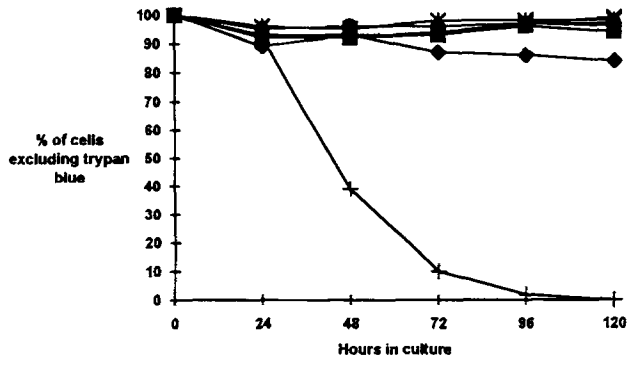

Figure 6. Exclusion of trypan blue by 14-d cord blood–derived hybrid granulocytes maintained for up to 5 d in RPMI 1640 with FCS alone (◆) or in RPMI 1640 supplemented with 0.01 (■), 0.1 (▲), 1 (×), 10 (*), and 100 (●) pM rhIL-5. The exclusion of trypan blue by freshly isolated peripheral blood eosinophils maintained in RPMI 1640 with FCS alone is indicated by (+).

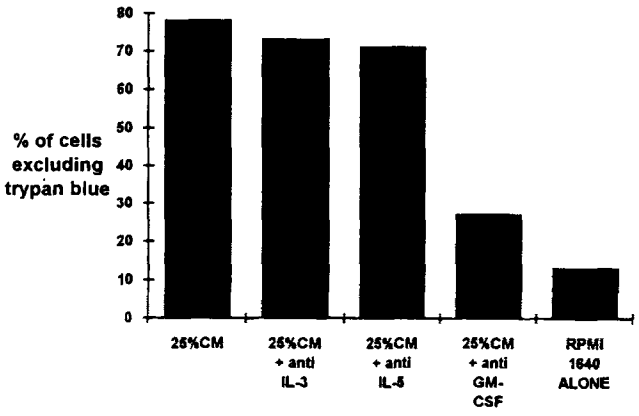

Figure 7. Exclusion of trypan blue by freshly isolated peripheral blood eosinophils cultured for 96 h in 25% conditioned medium (*CM*) from 14-d cord blood–derived cultured cells in the absence and presence of neutralizing antibodies against IL-3, IL-5, and GM-CSF. Right-hand column shows viability of eosinophils maintained in RPMI 1640 alone for 96 h.

three eosinophil hematopoietic cytokines (Fig. 7). 25% conditioned medium alone sustained the viability of 78 ± 3% of freshly isolated peripheral blood eosinophils after 96 h in culture. This proportion was not altered by the addition of 100 μg/ml antibody against IL-3 (73 ± 2% viable cells) or IL-5 (71 ± 1% viable cells). In contrast, 50 μg/ml of anti–GM-CSF antibody reduced the viable population to 27 ± 3% ($P < 0.01$) as compared to 13 ± 7% for eosinophils maintained in enriched medium lacking exogenous cytokines or conditioned medium ($n = 3$).

Discussion

This study of eosinophil development from cord blood precursors has unexpectedly revealed that a hybrid granulocyte with eosinophil and basophil granules predominated after cell culture for 14 d, and that these hybrid granulocytes are endowed with an autocrine viability-sustaining capacity by virtue of their elaboration of GM-CSF. The acquisition of granules by cord blood granulocyte precursors was accelerated by ~1 wk in cell populations that were developed on Matrigel in the presence of rhIL-3 and rhIL-5 when compared with replicate cells cultured in the presence of cytokines alone (Fig. 1). Furthermore, the phenotypic homogeneity was greatly increased by the presence of Matrigel. Indeed, staining with Wright's and Giemsa stains showed both eosinophilic and basophilic granules in 90% of these cells after 14 d in culture, as recorded in the time-dependent generation of these hybrids.

The predominant development by day 14 of granulocytes with hybrid eosinophil/basophil-staining characteristics was shown with Wright's and Giemsa staining. This was independently confirmed by the observation that 88% of these cells exhibited granule metachromasia when stained with toluidine blue and >90% contained a cyanide-resistant peroxidase, as shown for individual cells in Fig. 2, *B* and *C*. The recognition of granule markers specific for basophils (histamine) and for eosinophils (RNA transcripts encoding EDN) supports the presence of two granule phenotypes although it does not confirm their simultaneous occurrence in individual cells (Fig. 3). The hybrid nature of the granule population in the 14-d cultured cells was also confirmed by transmission electron microscopy, demonstrating the coexistence of immature eosinophil granules and basophil granules in the same cells (Fig. 4). Thus, the hybrid eosinophil/basophil granulocytes recognized in patients with acute and chronic myelogeneous leukemias (12, 14) are the predominant cellular phenotype in cultures of cord blood mononuclear cells maintained for 14 d in the presence of rhIL-3, rhIL-5, and Matrigel.

Denburg and colleagues (10) reported that clonally derived hematopoietic colonies grown from peripheral blood precursors in semisolid methylcellulose supplemented with Mo-conditioned medium contained mixtures of eosinophils and basophils. 5–48% of the cells in these colonies had both granule types. We observed that when exogenous cytokines and Matrigel were removed from the 14-d cultures and culture was continued in RPMI 1640 with 10% FCS alone for 6 d, 93% of the cells became eosinophils and this population exhibited a greater degree of maturity based on criteria of granule staining properties, cell size, and nuclear morphology than did replicate cells that were maintained in the presence of rhIL-3, rhIL-5, and Matrigel (Fig. 5). Those maintained with rhIL-3, rhIL-5, and Matrigel progressed to only 27% eosinophils, with 68% remaining hybrid granulocytes. There were not significant numbers of basophils in our culture system, either with or without exogenous cytokines. We therefore place these hybrids as intermediates capable of developing into mature eosinophils (Fig. 5).

The mechanism by which Matrigel exerted its effects on granule development is unclear. Both embryonic stem cells and mature eosinophils possess specific receptors for laminin, fibronectin, and other matrix components (30–32). The receptor-mediated interaction of hematopoietic stem cells with matrix components may result in signal transduction and expression of lineage-specific genes as demonstrated for mammary epithelium, hepatocytes, and other cell types (21–24, 33). Alternatively, extracellular matrix proteins can specifically bind and stabilize growth factors that may ensure the exposure of stem cells to optimal local concentrations of cytokines (34). Furthermore, nerve growth factor is a minor constituent of Matrigel. This cytokine has synergistic activity with GM-CSF (35) and with Mo-conditioned medium (36) in stimulating growth and differentiation of granulocyte colonies, particularly of the eosinophil/basophil lineage.

The progression of hybrid granulocytes to eosinophils after exogenous cytokines and Matrigel were removed from the cultures implies an autoregulatory capacity for maintenance of viability. As shown in Fig. 6, ~85% of these cells excluded trypan blue during the course of a 5-d experiment, and little difference in viability existed between cells maintained in the presence or in the absence of rhIL-5. We found that hybrid granulocyte-conditioned medium contained an eosinophil viability-sustaining activity for freshly isolated peripheral blood eosinophils and identified it immunochemically as bioactive GM-CSF (Fig. 7). 25% conditioned medium harvested from 14-d hybrid granulocytes after washing and culture for 48 h without added cytokines sustained the viability of peripheral blood eosinophils for 96 h at 78% as compared to 13% in RPMI 1640 with 10% FCS alone. The viability was reduced to 27% by neutralization with anti–GM-CSF antibody, a reduction of 78% in viability-sustaining activity, whereas anti–IL-3 or anti–IL-5 had no effect.

Mature eosinophils have been shown to produce GM-CSF under certain circumstances. A subset of eosinophils express GM-CSF mRNA in nasal polyps and in airway biopsies after segmental antigen challenge (37, 38). Furthermore, peripheral blood eosinophils can be induced to express GM-CSF protein by stimulation with calcium ionophore or by culture on fibronectin-coated plates (39–41). These observations have prompted speculation that certain eosinophils may have autocrine capabilities in inflammatory disease. Our observation that bioactive GM-CSF is constitutively expressed by hybrid granulocytes suggests that this cellular phenotype might be capable of regulating lineage viability as well as growth and

differentiation in an autocrine fashion. Furthermore, this phenotype may also prolong the viability and stimulate the function of other cells responsive to GM-CSF in a paracrine fashion.

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