Selective Stimulation by Mouse Spleen Cell Conditioned Medium of Human Eosinophil Colony Formation

By D. Metcalf, R. L. Cutler, and N. A. Nicola

Stimulation of un fractionated or nonadherent human marrow cells in agar culture by pokeweed-mitogen-stimulated BALB/c mouse spleen cell conditioned medium (SCM) led, in most cultures, to the exclusive formation of eosinophil colonies. The culture system exhibited linearity of eosinophil colony formation with varying numbers of cells cultured, and the absolute numbers and size of SCM-stimulated eosinophil colonies approximated those in cultures stimulated by human placental conditioned medium. The active factor in SCM for human eosinophil colony formation was not clearly separable from the factors stimulating granulocyte-macrophage and eosinophil colony formation by mouse marrow cells on ammonium sulfate and phenyl boronate chromatography, but was of larger size than the mouse-active factors and separable from them by phenyl sepharose chromatography. This selective culture system for eosinophil colony formation should be of value for studies on human eosinophil progenitor and maturing cell populations in a variety of disease states.

The growth of human eosinophil colonies in semisolid medium was first described by Chervenick and Boggs in autostimulated cultures of peripheral blood. Subsequent studies showed that comparable eosinophil colonies develop in cultures of human marrow or blood when stimulated by a variety of materials, including white cell underlayers or conditioned medium, and human placental conditioned medium. In all cases, eosinophil colonies are a minority subpopulation developing in the presence of larger numbers of neutrophilic and/or monocytic colonies. Although many eosinophil colonies have a characteristic tight appearance, not all are identifiable in unstained cultures, and the influence of other colony types of eosinophil colony formation is not known.

Attempts have been made to develop a system that selectively supports eosinophil colony formation by chemically fractionating the stimulus used. Although the active factor stimulating eosinophil colony formation (eosinophil colony-stimulating factor, EO-CSF) was shown to be of slightly larger molecular weight than GM-CSF, these attempts failed to separate EO-CSF from a subset of GM-CSF molecules.

Medium conditioned by lectin-stimulated mouse splenic lymphocytes has been shown to stimulate the formation by mouse bone marrow cells of eosinophil colonies as well as colonies containing neutrophils, macrophages, erythroid, and megakaryocytic cells. In general, murine CSFs fail to stimulate colony formation in vitro by human progenitor cells. However, in recent studies, addition of pokeweed-mitogen-stimulated mouse spleen cell conditioned medium (SCM) to human marrow cultures was observed to stimulate the formation of small numbers of colonies. Unexpectedly, these colonies were found to be composed exclusively of eosinophils.

The present studies were undertaken to amplify this observation and to determine whether mouse SCM is a reliable reagent for selectively stimulating the formation of eosinophil colonies by human progenitor cells.

MATERIALS AND METHODS

Bone Marrow Cultures

All cultures were performed in 35-mm plastic Petri dishes using 2 ml of Dulbecco's Modified Eagle's Medium containing 20% fetal calf serum and 0.3% agar. The formula of the medium has been described elsewhere.

Cultures contained 50,000 or 100,000 nucleated marrow cells obtained from rib segments removed during surgery on patients with no demonstrable hematologic abnormality or from marrow aspirates from patients undergoing investigation for nonleukemic disorders. All studies were performed after review and approval of the Ethics Committee of the Walter and Eliza Hall Institute.

Material to be tested for colony-stimulating activity was added in volumes of 0.1 or 0.2 ml to the empty culture dish before addition of the agar medium containing the marrow cells. After thorough mixing, the cultures were allowed to gel, then were incubated for 14 days in a fully humidified atmosphere of 10% CO₂ in air.

Scoring of Cultures

Colony counts were performed on cultures at day 14 of incubation, scoring as colonies all aggregates of more than 40 cells. All, or up to 45, sequential individual colonies were removed using a fine Pasteur pipette, placed on albumin-coated microscope slides, and stained with Luxol-Fast-Blue/hematoxylin. Typing of colonies was performed at 400× magnifications.

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Mice

Mice used were 3- to 6-month-old C57BL/6J/H-1 WEHI and BALB/c/An Bradford SEHI mice of either sex. All mice were raised under specific pathogen-free conditions in this Institute and held in conventional animal rooms for up to 4 wk before use.

Mouse Assay Cultures

Assays for granulocyte-macrophage and eosinophil colony formation stimulated by fractions of spleen conditioned medium were performed in 1-ml cultures containing 75,000 3- to 6-month-old C57BL femoral marrow cells in the above culture medium. In each case, 0.1 ml of test material was added to duplicate culture dishes before addition of the cell suspension in agar medium. All cultures were scored after 7 days of incubation and colony typing was performed on whole cultures fixed with glutaraldehyde then stained with Luxol-Fast Blue/hematoxylin.

Preparation of Conditioned Medium

Human placental conditioned medium (HPCM) was prepared from I-cm cubes of human placenta as described previously; the HPCM preparation used routinely was the post gel filtration stage (stage II).

Mouse spleen conditioned medium (SCM) was prepared by incubating 250-ml volumes of spleen cell suspensions in 500 ml capped Erlenmeyer flasks at 37°C in 10% CO2 in air for 7 days. The cell suspensions were from BALB/c mice aged 3 mo, and 2 x 10^5 cells/ml were cultured in RPMI 1640 medium containing 5 x 10^-5 M 2-mercaptoethanol, 5% heat-inactivated human serum, and 0.05 ml of a 1:15 dilution of pokeweed mitogen (GIBCO, Grand Island, NY) per ml of culture fluid. Culture media were harvested and cell debris removed either by centrifugation or by Millipore filtration.

Fractionation of Spleen Conditioned Medium

Spleen conditioned medium (in I.15-liter batches) was concentrated 23-fold using an Amicon DC-2 hollow fiber concentrator equipped with an H1 P10 cartridge having a molecular weight cut-off of 10,000.

All fractionation steps except gel filtration chromatography were performed at 20°C. Gel filtration chromatography was performed at 4°C. All fractionation buffers contained 0.02% Tween 20 (Sigma, St. Louis, MO) and 0.02% sodium azide.

Prior to assay, column fractions were diluted and exchanged into phosphate (0.02 M, pH 7.3) buffered saline (0.15 M) containing 0.02% Tween 20, using small columns containing Sephadex G-25M (PD-10 columns, Pharmacia, Piscataway, NJ) and then sterilized by passage through Millipore filters (0.45 μ).

Salting Out Chromatography

The concentrated SCM was dialyzed against 0.1 M sodium phosphate, pH 6.0, and was adjusted to 1 M ammonium sulfate by the addition of solid ammonium sulfate. This was then centrifuged at 12,000 g for 20 min to remove the small amount of precipitate that formed. The supernatant (49 ml) was slowly added to 60 ml of Sepharose CL-6B (Pharmacia) previously equilibrated in the same buffer. The mixture was stirred using an overhead stirrer, while 260 ml of 3.5 M ammonium sulfate in 0.1 M sodium phosphate, pH 6.0, was slowly added (20 ml/hr) using a peristaltic pump.

The gel, with bound protein, was recovered by filtration on a sintered glass filter and was washed with 2.8 M ammonium sulfate, 0.1 M sodium phosphate, pH 6.0. The washed gel was packed into a chromatography column (1.6 x 32 cm), and protein was eluted using a 400-ml decreasing gradient from 2.8 M to 0 M ammonium sulfate in 0.1 M sodium phosphate, pH 6.0, at a flow rate of 20 ml/hr. The gradient was monitored by measuring conductivity of fractions using a Philips conductivity meter (Philips, Australia). This step reduced the protein content of the pooled active fractions 2-5-fold with 80%–140% recovery of biologic activity.

Phenyl Boronate Chromatography

Active fractions from salting out chromatography were pooled (71 ml), adjusted to pH 8.5 by the addition of 1 M glycylglycine, pH 10.0, and applied to a column (0.9 x 28 cm) of phenyl boronate-agarose, (PBA 30, Amicon Corporation, Danvers, MA), previously equilibrated in 50 mM glycylglycine, pH 8.5. The column was washed with equilibration buffer until no protein was detectable in the eluant, and then bound protein was eluted by a linear gradient of 0-20 mM sorbitol in 50 mM glycylglycine, pH 8.5, at a flow rate of 8 ml/hr. This fractionation procedure was used for analytical purposes only, but the protein content of pooled active fractions was reduced twofold, with approximately 80% recovery of biologic activity.

Gel Filtration Chromatography

Active fractions eluted from PBA 30 by sorbitol were concentrated over a YM-10 membrane (Amicon Corporation) using a stirred cell concentrator. The concentrate (7.5 ml) was then applied to a column of Ultrogel AcA45 (2.5 x 88 cm) (LKB, Sweden), equilibrated in phosphate-buffered saline. The column was then eluted with phosphate-buffered saline at a flow rate of 20 ml/hr. Molecular weight was estimated from a linear plot of log molecular weight versus distribution coefficient using bovine serum albumin (67,000), ovalbumin (45,000), α-chymotrypsinogen (26,000), and cytochrome-c (13,000) as standards. This step resulted in an 80-fold reduction in the protein content of pooled active fractions, with a 50% recovery of biologic activity.

Phenyl-Sepharose Chromatography

Pooled fractions from salting out chromatography were applied directly to a column (2.6 x 39 cm) of phenyl-Sepharose CL-4B (Pharmacia) previously equilibrated with 1.5 M ammonium sulfate in 0.1 M sodium phosphate buffer, pH 6.0. The column was eluted with a linear gradient (400 ml) from equilibration buffer to 0.1 M sodium phosphate buffer. pH 6.0, at a flow rate of 20 ml/hr. The gradient was determined by measuring the conductivity of fractions. This step was associated with a sevenfold reduction in protein content of pooled mouse-active fractions, but no significant reduction in the protein content of human-active fractions.

Adherent Cell Removal

Adherent and phagocytic cells were removed from marrow cell suspensions using the carbonyl iron technique described by Lundgren et al.

RESULTS

Cultures of 50,000 or 100,000 human marrow cells incubated for 14 days with 0.1 ml of unfraccionated mouse spleen conditioned medium (SCM) developed small compact colonies of opaque cells similar in general appearance to eosinophil colonies stimulated by human placental conditioned medium (HPCM). Examination of stained colonies confirmed that they were composed wholly of Luxol-Fast-Blue-positive
peripheral blood cells or HPCM and containing colonies of other cell types.

The remarkable feature of SCM-stimulated cultures was the usual absence of neutrophilic and/or monocytic colonies (Table 1). Furthermore, the cultures also contained very few clusters of any type between the colonies. The absence of other colonies in SCM-stimulated cultures was invariable, unless the cultured marrow developed small numbers of monocyte-macrophage colonies in unstimulated cultures. In the latter case, similar small numbers of monocyte-macrophage colonies developed in SCM-stimulated cultures. In an analysis of cultures of 17 different marrow specimens (Fig. 2), 100% of colonies were eosinophilic in 14 instances, and in the other 3, eosinophilic colonies comprised more than 90% of the colonies developing. The remaining small monocytes colonies were readily distinguishable by their dispersed shape and the large size of colony cells. In control cultures of the same bone marrow stimulated by HPCM, eosinophil colonies comprised an average of 30% ± 19% of colonies present in the cultures.

Macroage colony formation in SCM-stimulated cultures was prevented or minimized by use of nonadherent marrow cells or by culturing fewer than 50,000 cells per dish. Neither procedure influenced the development or size of eosinophil colonies in SCM-stimulated cultures.

In SCM-stimulated cultures, a linear relationship was observed between the number of nonadherent cells cultured and the number of eosinophil colonies developing.

Fig. 1. Montage showing three typical Luxol-Fast-Blue-positive eosinophil colonies from 14-day cultures of human nonadherent marrow cells stimulated by pokeweed mitogen-stimulated mouse spleen conditioned medium.

eosinophils (Fig. 1). The eosinophil colonies in SCM-stimulated cultures exhibited a moderate variability in size, but no more so than that seen in conventional human marrow cultures stimulated by peripheral blood underlayers or HPCM. In some experiments, the average size of the eosinophil colonies in SCM-stimulated cultures was similar to that of colonies in HPCM cultures. However, in many cases, colony size was somewhat smaller than that seen in cultures maximally stimulated with HPCM or white cell underlayers. Although most SCM-stimulated eosinophil colonies were compact, often the colonies were loosely dispersed or had a tight center with a mantle of dispersed cells. In each case, the colonies were entirely composed of eosinophils but the variation in colony shape emphasized the known technical difficulty in identifying all eosinophil colonies with certainty in unstained cultures stimulated by underlayers of human marrow cultures stimulated by peripheral blood cells or HPCM and containing colonies of other cell types.

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Table 1. Selective Stimulation of Eosinophil Colony Formation by Nonadherent Human Marrow Cells
Using Mouse Spleen Conditioned Medium

<table>
<thead>
<tr>
<th>Number of Cells Cultured</th>
<th>SCM</th>
<th></th>
<th>HPCM</th>
<th></th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean No. * of Colonies</td>
<td>Total Colonies</td>
<td>Mean No. of Colonies</td>
<td>Total Colonies</td>
<td>Mean No. of Colonies</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>GM</td>
<td>M</td>
<td>EO</td>
<td>G</td>
</tr>
<tr>
<td>100,000</td>
<td>22 ± 9</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>11</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
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<td>2 ± 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Cells cultured for 14 days in the presence of 0.2 ml mouse spleen conditioned medium (SCM), 0.2 ml human placental conditioned medium (HPCM), or 0.2 ml saline.

*Mean colony counts ± standard deviations from four replicate cultures.

±Absolute numbers of different types of colonies calculated from differential counts, where possible, on 30 sequential colonies stained with Luxol-Fast-Blue/hematoxylin. G, neutrophilic granulocytic; GM, neutrophilic granulocyte-monocytic; M, monocytic; EO, eosinophil colonies.

spleen conditioned medium appeared to stimulate maximal numbers of eosinophil colonies to develop, but further studies are required using concentrates of such material to determine whether all available eosinophil progenitors are stimulated by the use of unconcentrated SCM.

Since SCM is prepared routinely using 5% human serum, it was possible that the human serum used might have been responsible for the observed eosinophil colony formation. Tests on sera from a variety of patients showed, in agreement with previous studies, that an occasional serum was able to stimulate granulocyte-macrophage and eosinophil colony formation by unfractionated bone marrow cells, but in no case was a pure population of eosinophil colonies observed. No colony formation of any type was observed with a final concentration of human serum corresponding to that present in SCM-stimulated cultures.

To further eliminate the possible role of serum in the observed activity of SCM, six special batches of pokeweed-mitogen-stimulated spleen conditioned medium were prepared using RPMI 1640 medium without serum and containing 2–10 x 10⁶ cells/ml. Tests using these serum-free SCM preparations showed that each was able to selectively stimulate eosinophil colony formation in cultures of human marrow cells, although the overall level of activity was slightly lower than with conventional SCM.

**Fractionation of SCM**

Analysis of the activity of fractions of SCM separated by ammonium sulfate chromatography (Fig. 5) indicated that the activity stimulating human eosinophil colony formation was nearly indistinguishable from activity stimulating colony formation from murine adult bone marrow based on the relative solubility in ammonium sulfate (2–1.3 M). Mouse eosinophil colonies are not plotted separately in Fig. 5, but represented 5%–10% of mouse colonies in each active fraction. Active pools from this step were chromatographed on a phenyl-boronate/agarose column (Fig. 6), which recognizes cis diol moieties on mannose and sialic acid carbohydrate residues. Because of the high ammonium sulfate concentration in the SCM pool, most of the protein and CSF activities initially bound
Fig. 4. Absolute number of eosinophil colonies developing in cultures of 20 different human marrow specimens stimulated by human placental conditioned medium (HPCM) or mouse spleen conditioned medium (SCM). Lines join data from same marrow specimen.

Fig. 5. Fractionation of mouse spleen conditioned medium using ammonium sulfate salting out chromatography. Note coincidence of peak of human eosinophil colony-stimulating activity (■■) with peaks of colony-stimulating activity for total mouse granulocyte-macrophage and eosinophil colonies (○○). The ammonium sulfate concentration is indicated by the dashed line.

Fig. 6. Fractionation of mouse spleen conditioned medium by phenyl-boronate chromatography using pooled active fractions from Fig. 5. Note coincidence of peaks of human eosinophil colony-stimulating activity (■■) with peaks of colony-stimulating activity for total mouse granulocyte-macrophage and eosinophil colonies (○○). The sorbitol concentration is indicated by the dashed line. The arrow indicates the point at which elution with 50 mM glycylglycine, pH 8.5, was begun.

DISCUSSION

Mouse spleen cell conditioned medium (SCM) appears to be a reliable reagent for selectively stimulating eosinophil colony formation by human marrow cells. The size of the colonies developing is often slightly smaller than that of eosinophil colonies in cultures stimulated by human white cell underlayers or placental conditioned medium. However, the absolute selectivity for eosinophil colony formation makes the...
Fig. 7. Fractionation of mouse spleen conditioned medium (active pool from salting out chromatography) on phenyl-Sepharose CL-4B using a decreasing linear gradient of ammonium sulfate for elution. Fractions were assayed for (O—O) total murine bone marrow colonies, (X—X) Luxol-Fast-Blue-positive murine eosinophil colonies, and (Δ—Δ) Luxol-Fast-Blue-positive human eosinophil colonies stimulated in cultures of human bone marrow.

SCM-stimulated culture system ideal for studies on proliferating populations of human eosinophil precursors or for functional studies on pure eosinophil populations where living cells need to be harvested for further analysis. For example, the system should be of value in investigating whether eosinophil precursors are part of the neoplastic clone in AML and CML and for investigating the nature and function of eosinophil populations in a wide variety of diseases involving eosinophils.

From the biochemical data available so far, it appears that the factor in SCM stimulating human eosinophil colony formation is different from the factors stimulating granulocyte-macrophage or eosinophil formation by mouse progenitor cells. The difference was most evident using phenyl-Sepharose chromatography. The higher apparent molecular weight of the human-active eosinophil factor is of interest, since EO-CSF from human sources (placental conditioned medium) has a similar apparent molecular weight to that found here for human-active material in SCM. It is of interest that human eosinophil progenitors are responsive to a factor in mouse SCM since, in general, human granulocyte-macrophage progenitor cells are unresponsive to colony-stimulating factors of murine origin. It is puzzling why mouse spleen cells should synthesize a human-active eosinophil colony-stimulating factor that appears to be unable to stimulate the proliferation of corresponding mouse cells. However, colony-stimulating factors also stimulate end-cell functional activity, and it is worth testing whether the human-active factor is able to stimulate the functional activity of mature mouse eosinophils.

From the linearity of colony formation with cultured cell numbers and the activity of SCM on nonadherent cells, the human-active factor in mouse SCM appears to act directly on human eosinophil progenitors. Parallel studies have shown that mouse SCM is able to stimulate the functional activity of preformed mature human eosinophils as effectively as human-derived EO-CSF (Vadas M, Nicola NA, and Metcalf D, unpublished data), suggesting that the reactivity of human cells to the human-active material in mouse SCM extends to the most mature cells in the human eosinophilic series.

This culture system appears to be a reliable method for selectively growing colonies of eosinophils for further study and for analyzing the frequency and nature of human eosinophil progenitor populations in clonal cultures free of any interactions with more numerous granulocyte-monocyte or erythroid colonies.

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