Stimulation of Eosinophil Production In Vitro by Eosinophilopoietin and Spleen-Cell-Derived Eosinophil Growth-Stimulating Factor

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Eosinophilopoietin (EPP) was previously characterized by the ability to stimulate eosinophil production in vivo, but these studies could not ascertain whether EPP had a direct effect on the bone marrow or acted indirectly by causing release of eosinophilopoietic activity by other tissues. The present studies demonstrate that EPP stimulates eosinophil growth in liquid culture of mouse bone marrow in vitro. The timing of stimulation by EPP in vivo and in vitro were parallel, with maximal eosinophil growth after 48 hr. Moreover, EPP appears similar to, and possible identical with, the eosinophil growth-stimulating substance (EO-GSF) released by antigenic stimulation of immune nonadherent spleen cells. Both EPP and EO-GSF are of low molecular weight, both produce stimulation of eosinophil growth with identical kinetics, and both produced similar dose-response curves in the liquid culture system.

The search for humoral substances that control growth and differentiation of hematopoietic cells has recently been facilitated by development of quantitative techniques for studying growth of bone marrow cells in vivo and in vitro. Substances that promote production of neutrophil granulocyte or monocyte-macrophage cell lines are released by diverse tissues. The substances regulating the growth of eosinophils are less well defined. Studies of eosinophilopoiesis in vivo have utilized two seemingly disparate approaches. One method has been to deplete circulating eosinophils with specific antiserum and then to examine the sera of such animals for eosinophilopoietic activity. The other approach has sought the release of stimulatory activity following antigenic challenge of immune cells, especially T lymphocytes.

Depletion of mature, circulating eosinophils with antieosinophil serum (AES) was accompanied by an increase in immature eosinophils in the bone marrow. Serum from such AES-treated mice contained a low molecular weight, pronase-sensitive substance that induced increased numbers of total and dividing bone marrow eosinophils and total circulating eosinophils when injected into normal mice. This suggested a true poietic activity in vivo, and the responsible substance was termed “eosinophilopoietin” (EPP). As this EPP activity has only been shown in vivo, its effect on bone marrow cultures in vitro is unknown. Furthermore, whether EPP acts directly to stimulate immature eosinophil precursors or stimulates another cell population to release a substance that then acts on the eosinophil series has not been evaluated. Recent studies employing athymic nude mice suggest that an intact T-lymphocyte population is required for the generation of EPP but is not necessary for the stimulation of eosinophil production by preformed EPP.

Immune stimulation of the eosinophil granulocyte series in vivo also appears limited to systems that are mediated by, or dependent on, T lymphocytes. The eosinophilias produced by trichinosis, schistosomiasis, or alum-precipitated tetanus toxoid are dependent on an intact T-lymphocyte population. In these models antigen-specific stimulation of T lymphocytes causes release of soluble substance(s) that stimulates eosinophilopoiesis in vivo, but the stimulatory factor(s) has not been characterized. Whether these factors stimulate eosinophilopoiesis directly, or cause other cells to release substances with such a direct effect, and/or cause changes in the hemopoietic microenvironment to enhance eosinophil production is also uncertain.

Studies of eosinophilopoiesis in vitro have employed semisolid media (agar or methylcellulose) or liquid culture. In semisolid media, “eosinophil colony-stimulating factor” (EO-CSF) induces the division and differentiation of cells at a stage before the myeloblast (and recognizable as eosinophils) to form colonies of recognizable eosinophils. Antigen or mitogen-stimulated lymphocytes appear to provide the major source of EO-CSF. Purification of EO-CSF has been elusive; however, it is distinct from neutrophil/macrophage CSF by electrophoretic mobility and antigenici-
vitro effects of EPP on bone marrow cultures and the relationship of EPP to a lymphocyte-derived stimulator of eosinophil growth in vitro, EO-GSF. We demonstrated that EPP was capable of promoting eosinophil proliferation in mouse bone marrow during in vitro liquid culture, that the kinetics of eosinophil proliferation induced by EPP or by EO-GSF were similar, and that both EPP and EO-GSF are of low molecular weight.

MATERIALS AND METHODS

Animals

Young adult CF-1 mice (Carworth, N.Y.), 18–22 g, were used as the source of serum EPP. Either 8- to 10-wk-old C3H/HeJ (Jackson Laboratories, Bar Harbor, M.) or 7- to 9-wk-old ICR-CD1 (Charles River Farms, Mass.) male mice were used, as indicated, as the source of bone marrow and/or spleen cells.

Eosinophilotpeitin

EPP-rich mouse sera were induced and fractionated as previously described.4 Groups of CF-1 mice were injected i.p. 3 times at 48-hr intervals with 0.25 ml of antieosinophil serum (AES) or normal rabbit serum (NRS). Pooled sera were obtained 72 hr after the last injection. Aliquots of 1 ml serum from animals treated with AES or NRS were subjected to gel filtration on sephadex G-25 and 1-ml fractions were collected. Fractions collected between two markers, vitamin B12 (molecular weight 1357) and 14C-histamine (molecular weight 186) were pooled, lyophilized, and reconstituted with sterile distilled water.

In Vivo Assay for EPP

Stimulation of eosinophil growth in vivo was examined as described previously.4 Reconstituted fractions (corresponding to 0.25 ml of mouse sera) were injected intravenously into groups of normal mice. Femoral bone marrow was flushed after 48 hr, and eosinophil numbers were determined.

Trichinella spiralis Antigen: Excretion-Secretion Products of Larvae (L[ES]).

T. spiralis was maintained by serial infection of C3H/HeJ mice. Muscle stage larvae were isolated by acid-pepsin digestion of infected musculoskeletal tissues and were incubated in phosphate-buffered saline (PBS), pH 7.2, to produce an L(ES)-rich solution as described previously.18,19 Briefly, larvae were freed of bacterial contamination by a 30-min exposure to an antibiotic/merthiolate/PBS solution,20 washed thoroughly in PBS alone, and adjusted to 2.5 × 105 larvae/ml in PBS containing 500 U of penicillin (Gibco, Grand Island, N.Y.) and 500 µg streptomycin (Sigma Chemical Co., St. Louis, Mo.). Larvae (2.5 × 105) were then incubated for 9 hr at 37°C in a 50-ml erlenmeyer flask. The medium was centrifuged at 1500 g for 30 min, concentrated by ultrafiltration (UM-05, Amicon, Lexington, Mass.), and frozen (−70°C). Prior to use, the protein concentration was determined according to the “Bio-Rad Procedure”.21

Preparation of Spleen Cell Conditioned Medium (L-CM)

Conditioned medium was obtained as the supernatant of cultures of sensitized, nonadherent spleen cells with excretory-secretory antigens of trichinella larvae.18 Single cell suspensions were prepared from spleens of mice 4–6 wk after infection with trichinosis. Adherent cells were removed by 3 successive 1.5-hr incubations of 5 × 106 cells in plastic dishes (Falcon Plastics, Oxnard, Calif.). The nonadhering cells consisted of 90% lymphocytes as determined by May-Grünwald-Giemsa staining and cytochemical demonstration of peroxidase22 and nonspecific esterase.23 These cells were incubated at 2.0 × 106 cells/ml in CMRL-1066 medium (Gibco) supplemented with 5% FCS, 0.35 mM L-asparagine (Sigma), 0.001 mM vitamin B12 (hydroxycobalamine, Merck and Co., Pa.), 100 µg streptomycin (Sigma), and 150 U penicillin/ml (Gibco). They were stimulated by the addition of the optimal dose of L(ES), 10−3 µg protein/ml, as determined in earlier experiments.24 The cell-antigen mixture (1 ml) was incubated in 17 × 10 mm polypropylene culture tubes (Falcon) at 37°C in a humidified 5% CO2 atmosphere for 1–5 days. The conditioned medium (L-CM) was filtered (0.45 µm, Millipore), cooled to 4°C, and assayed for growth factors within 24–96 hr. In certain experiments, preparations of L-CM were subjected to ultrafiltration employing a filter (DM-5, Amicon) with a 5000-dalton exclusion. The stimulatory activity of the L-CM and ultrafiltration filtrate were then compared.

In Vitro Assay of Eosinophil Production

The ability of test substances to promote eosinophil proliferation of normal, syngeneic, or allogeneic femoral bone marrow was assayed in a liquid culture system similar to that of Sumner et al.25 Bone marrow cells from two femurs were suspended in CMRL-1066 supplemented with 10% FCS, 10% tryptose phosphate (Difco Laboratories, Detroit, Mich.), 5% horse serum (Gibco), 1.0 mM pyruvic acid, and L-asparagine, vitamin B12, and penicillin as above.18 Cell viability was determined by trypan blue exclusion. Adherent cells were removed by incubating 2 × 105 viable cells in 60-mm plastic tissue culture dishes (Falcon) for 2 hr. The nonadhering cell fraction was diluted to a viable cell concentration of 2.4 × 106/ml. Fluids containing putative stimuli or control preparations were assayed in a blind manner. These preparations were added to a final concentration of 10% or less as indicated. One-milliliter aliquots of the bone marrow nonadherent cells were cultured in upright, 10-ml polypropylene tubes (Falcon). Five culture tubes were used per sample. After appropriate intervals, cell aliquots were taken and dispersed by pipette (1 ml) and needle (26 gauge) tituration. Duplicate aliquots were diluted 1:2 in Rud’s eosinophil stain27 modified as follows: 0.2 g phloxine B (Roboz Inc., Washington, D.C.), 20 ml aceton, 35 ml distilled water, and 5 ml 1% NaCO3. Eosinophils were counted in large volume hemocytometers (Speirs-Levy eosinophil counting slide, C.A. Hauser and Son, Pa.). Total viable and differential cell counts were also performed.

RESULTS

In Vitro Effect of EPP on Mouse Bone Marrow Cultures

Low molecular weight fractions obtained from the serum of mice pretreated with antieosinophil serum (MS-AES) stimulated eosinophil growth in the in vitro assay. A 4–10-fold increase of numbers of eosinophils was evident after 48 hr (Fig. 1 and 2). Low molecular weight fractions obtained from the serum of mice pretreated with normal rabbit serum (MS-NRS) (Fig. 1 and Table 1) or higher molecular weight fractions from MS-AES (elution of 40%–55% of the
sephadex G-25 bed volume) did not stimulate eosinophil growth (Fig. 2 and Table 1). Stimulation of eosinophil production was related to the quantity of MS-AES added to the bone marrow cultures (Figs. 3 and 4). The activity of MS-AES and control preparations in vivo was similar to that observed during in vitro bone marrow cultures (Table 1).

**In Vitro Effect of L-CM**

Conditioned medium containing substances released by antigen-stimulated nonadherent spleen cells (L-CM) also caused a rapid increase in numbers of eosinophils in vitro, and the kinetics paralleled those produced by MS-AES fractions (Fig. 1 and 2).

The eosinophil stimulatory activity in L-CM appears to be of low molecular weight, as judged by ultrafiltration experiments. Two preparations of L-

<table>
<thead>
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<th>Preparation</th>
<th>Sephadex Fraction</th>
<th>In Vivo Eosinophils/10^5/Femur</th>
<th>In Vitro Eosinophils/ml</th>
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<tr>
<td>1</td>
<td>MS-AES 60-100</td>
<td>2.6 ± 0.2 (4)*</td>
<td>19 ± 1.3 (4)</td>
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<td></td>
<td>MS-NRS 60-100</td>
<td>1.6 ± 0.2 (4)</td>
<td>9.5 ± 1.2 (4)</td>
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<td></td>
<td>Medium alone</td>
<td>—</td>
<td>7.8 ± 0.85 (4)</td>
</tr>
<tr>
<td>2</td>
<td>MS-AES 60-83</td>
<td>5.2 ± 0.6 (6)</td>
<td>22 ± 1.5 (6)</td>
</tr>
<tr>
<td></td>
<td>MS-AES 40-55</td>
<td>2.8 ± 0.2 (6)</td>
<td>10.3 ± 1.1 (6)</td>
</tr>
<tr>
<td></td>
<td>Medium alone</td>
<td>—</td>
<td>9.0 ± 1.03 (6)</td>
</tr>
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*Mean ± SEM of (n) determinations made 48 hr after administration of putative stimulant.
Fig. 3. In vitro production of normal C3H/HeJ bone marrow eosinophils in vitro as a function of MS-AES concentrations. (A and B) Inactive and active fractions of MS-AES as in Fig. 2. L-CM and PBS-control are conditioned medium and control medium at 10% concentration, as in Fig. 1.

Fig. 4. In vitro production of normal C3H/HeJ or ICR-CD1 bone marrow eosinophils as a function of decreasing concentration of MS-AES or L-CM. Each preparation was cultured for 4 days and yielded growth curves similar to those in Fig. 3. This figure depicts the net stimulation (stimulated eosinophils/ml less the eosinophils/ml in the medium control) observed after 48 hr of culture.

CM were passed through a membrane with a 5000 molecular weight exclusion. The L-CM, filtrate, retentate, and the CMRL 1066 medium were assayed in the in vitro marrow culture; the numbers of eosinophils/ml (x 10^3) after 48 hr of incubation were as follows: L-CM, 21.5 ± 0.84 (n = 6); filtrate, 18.6 ± 0.72 (n = 6); medium 7.0 ± 0.41 (n = 4).

The activities of both MS-AES and L-CM were dose-related (Figs. 3 and 4). Similar decreases in relative concentrations yielded similar decreases in stimulation of eosinophil production. There may be variations in the sensitivity of different strains of mice to these substances, as the bone marrow of C3H/HeJ mice appeared considerably less sensitive than bone marrow obtained from ICR-CD1 mice (Fig.4).

**DISCUSSION**

The successful culture of bone marrow cells has resulted in the discovery of several substances that stimulate proliferation of granulocytes in vitro. Attempts to examine the roles of these substances in vivo have, however, been few and inconclusive. For example, Metcalf and Stanley used human urine as a source of partially purified colony-stimulating factor, a substance that stimulates growth of granulocytes and macrophages in semisolid medium; injection of this human CSF into mice produced a neutrophilia and monocytosis and an apparent increase in granulopoiesis. They could not be certain if the response was due to a nonspecific inflammatory response to foreign proteins, due to the impurity of the material injected, or due to a true in vivo granulopoietic effect of the CSF.

In contrast, the eosinophilopoietic effect of EPP was first defined in vivo. The present studies demonstrated that EPP stimulated eosinophil production in vitro with kinetics identical with the activity of EPP in vivo. Stimulation of eosinophilopoiesis was rapid, as eosinophil production was maximal after 48 hr. This was followed by a rapid return (within the following 48 hr) to normal levels. The rapidity of the response strongly suggests that the effect involved a direct stimulation of eosinophil precursors. Thus, EPP may be a true granulopoietic factor in that it has similar stimulatory effects both in vivo and in vitro.

Stimulation of eosinophilopoiesis during in vivo responses to various antigens has been shown to be mediated by T lymphocytes. EO-GSF is an eosinophil growth-stimulating substance that is released by antigen-stimulated nonadherent spleen cells, probably lymphocytes. The stimulatory effects of EO-GSF are specific for the eosinophil leukocytes. The gener-
ation of EPP is also dependent on an intact T-lymphocyte population. These observations raised the question of the relationship between EPP and EO-GSF. The present studies suggest that they are similar, and perhaps identical, substances. Both are of low molecular weight and the kinetics of their in vitro effects were remarkably parallel. Both demonstrated similar dose-response curves in the liquid culture assay. Although lower concentrations of each produced less stimulation of eosinophil production, the kinetics of the eosinophil responses were not changed.

Antigen-mediated stimulation of eosinophil growth in vivo has an onset that is similar to that observed in the liquid culture systems stimulated by EPP or EO-GSF. Production of bone marrow eosinophilia by sensitized mice challenged with appropriate antigens would require time for lymphocyte release of stimulatory activities and for the appropriate bone marrow responses. Sensitized mice challenged with i.v. injection of muscle-stage larvae of T. spiralis, alun-precipitated tetanus toxoid, or schistosome egg antigen developed bone marrow eosinophilia that was evident 3–4 days after challenge. Furthermore, thoracic duct lymphocytes, isolated following stimulation by infestation with T. spiralis, induced a peripheral blood eosinophilia within 2 days after i.v. administration to normal mice. The kinetics of these responses are compatible with the hypothesis that EPP/EO-GSF are involved in the initiation of the eosinophilopoietic response in vivo.

The relationship of EPP and EO-GSF to colony-forming factor (EO-CSF), which is assayed by colony formation in semisolid medium, needs further investigation. Although EO-CSF is also released by lymphocytes, murine EO-CSF has a molecular weight of approximately 50,000. Moreover, whereas EPP and EO-GSF cause a rapid stimulation of eosinophil production, EO-CSF produces eosinophil growth that is maximal after 5–14 days of culture. In other studies of EO-GSF, we have shown that the rapid cessation of eosinophilopoiesis is due to depletion of cells responsive to EO-GSF. Addition of fresh EO-GSF to cultures after 48 hr of stimulated growth did not cause further eosinophil production; the numbers of eosinophils decreased in parallel with cultures unexposed to a second stimulus. Conversely, the EO-GSF activity of the culture fluid remained essentially unchanged after 48-hr culture, as shown by a test on fresh marrow cells. It is tempting to speculate that the distinctive activities of EPP/EO-GSF and EO-CSF are due to different substances that act at different stages of differentiation of eosinophil leukocyte series.

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