TWO TYPES of culture systems have been used to measure hemopoietic cell proliferation. In one type, hemopoietic cells are suspended in a semisolid medium where discrete colonies result from the proliferation of progenitor cells. 1 Factors that stimulate these "colony-forming cells" to proliferate are given the operational definition "colony-stimulating factor" or CSF. In the second type of culture, hemopoietic cells are suspended in liquid. 2 In this type, the levels of specific cell types are determined as a function of time. A factor that stimulates cells to proliferate under this condition is known as a "growth-stimulating factor" or GSF.

Eosinophil CSF (Eo-CSF) is assayed by determining the number of eosinophil colonies produced in semisolid medium. These colonies are detected by in situ observation and staining. 1 Eosinophil GSF (Eo-GSF) is assayed by determining the number of eosinophils in aliquots of liquid cultures exposed to specific stains (see Materials and Methods).

Direct evidence of the separate identity of eosinophil and neutrophil/macrophage factors has been difficult to obtain. Antigen- or mitogen-stimulated lymphocytes released various colony- and growth-stimulating factors, 1,3,4 but eosinophil CSF (Eo-CSF) was difficult to isolate. 5 The electrophoretic mobility of Eo-CSF, however, differed from neutrophil/macrophage CSF (GM-CSF), and antiserum raised against GM-CSF suppressed GM, but not eosinophil colony formation. 5

Ruscetti et al. 6 showed that eosinophil levels were increased in liquid cultures exposed to conditioned medium derived from cultures of sensitized spleen cells exposed to Trichinella spiralis antigen. In the current study, we have extended that observation and showed that Eo-GSF and other GSFs are differentially released from such spleen cells.

MATERIALS AND METHODS

Animals

Seven to nine-wk-old ICR-CD1 (Charles River Farms, Mass.) and 8-10-wk old C3H-HeJ (Jackson Laboratories, Bar Harbor Me.) male mice were used, as indicated, throughout the study.

T. spiralis Excretion-Secretion Products of Larvae [L(ES)]

Muscle stage larvae from C3H-HeJ mice were incubated in phosphate-buffered saline (PBS, pH 7.2) to produce an L(ES)-rich solution as described previously. 1 Briefly, larvae were freed of bacterial contamination by a 30-min exposure to an antibiotic/merthiolate/PBS solution, 4 washed thoroughly in PBS alone, and adjusted to 2.5 x 10^3 larvae/ml in PBS containing 500 U of penicillin (Gibco, N.Y.) and 500 μg streptomycin (Sigma, St. Louis, Mo.). Larvae (2.5 x 10^4) were then incubated for 9 hr at 37°C in a 50-ml Erlenmeyer flask. The medium was centrifuged 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." 5

Preparation of Spleen Cell Conditioned Medium [L(ES)-CM]

Mice were infected with 200 larvae of T. spiralis and aseptically splenectomized 4-6 wk postinfection. Minced spleens were pressed through a stainless steel sieve into cold CMRL-1066 medium (Gibco, N.Y.) supplemented with 5% fetal calf serum (FCS) (Gibco, N.Y.). Cells were then gently passed through decreasing sizes of syringe needles (22g-26g) to obtain a monodispersed suspension. Adherent cells were removed by incubating 5 x 10^5 cells/100 mm dish (Falcon Plastics, Calif.) for 3 successive 1.5-hr periods at 37°C in a humidified 5% CO_2 atmosphere. Nonadhering (NA) cells were removed after each period. The NA population consisted of 90% lymphocytes as determined by May-Grünwald-Giemsa staining and cytochemical demonstration of peroxidase and nonspecific esterase. 11 The NA cells were washed with PBS (Sigma, St. Louis, Mo.), resuspended, and stimulated by the addition of 10 μg/ml protein L(ES). The dose of antigen is critical to growth factor release 5 and was determined in earlier experiments. 12 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. Conditioned medium (CM) was passed through a sterile filter (0.45-μm, Millipore), cooled to 4°C, and assayed for growth factors within 24–96 hr.

**Assay for Eo- and GM-GSF**

Marrow cells (1 ml) were cultured in polypropylene tubes (10-ml capacity, Falcon) essentially as described by Sumner et al. Bone marrow of two femurs was obtained as described. Mononuclear suspensions were obtained by passage of cells through decreasing sizes of needles and 2 × 10⁷ viable cells (as determined by trypan blue exclusion, 0.05%) were incubated in a 60-mm dish (Falcon) for 2–4 hr in a humidified, 5% CO₂ atmosphere at 37°C. Nonadherent cells were removed and diluted to 2.5 × 10⁷ nucleated cells/ml in CMRL-1066 supplemented with 10% L(ES)-CM, 10% tryptose phosphate broth (Difco, Mich.), 5% horse serum (Gibco, N.Y.) and pyruvic acid, l-asparagine, and antibiotics as described for the lymphocyte medium. The Eo-GSF was assayed by its ability to stimulate eosinophil proliferation in normal syngeneic marrow cells of uninfected mice. Six cultures were set up and aliquots (0.3 ml) were removed from 2 tubes at daily intervals for cell counting. Cells were dispersed by titration in a pipette (1 ml) followed by a needle (26g) prior to sampling. The sample was diluted 1:1 with a micro-liter pipette (Ranin Inst., Mass.) in Rud’s eosinophil stain modified as follows: 0.2 g phloxine B (Roboz Inc., Washington, D.C.), 20 ml acetone, 75 ml distilled water, and 5 ml sodium bicarbonate (1%). Eosinophil granules stained deeply within 5 min, and eosinophils were counted in large volume hemocytometers (“Spiers-Levy eosinophil counting slide,” C.A. Haussen and Son, Pa.). Two other cell samples were taken and used in determining the total number of cells and the leukocyte differential, respectively. The leukocyte differential was performed according to standard procedure and the values obtained used in determining the number of cells of each series. Cell smears were obtained with a cytocentrifuge (Cytospin, Shanden) and stained with May-Grünwald-Giemsa.

**RESULTS**

Increased numbers of eosinophils were produced within 1 day when marrow cells were exposed to day-5 conditioned medium [L(ES)-CM] (medium obtained from 5-day-old cultures of sensitized spleen cells exposed to the excretory-secretory products of *T. spiralis* larvae, 10⁻³ μg/ml; Fig.1). The rate of increase was rapid but maintained briefly, 2–3 days. Beginning at 2–3 days, eosinophil numbers fell abruptly and reached control levels at 3–4 days. A dilution analysis of the CM showed that, based both on growth rate and growth potential, the assay provided a relative indication of the amount of Eo-GSF present (Fig. 2). The total number of viable cells, however, fell sharply for 2 days and began to increase between 4 and 5 days. High levels were still present at 8 days (Fig. 1). In an independent experiment, neutrophil and monocyte/macrophage production was measured (Fig. 3). Neutrophils rapidly increased in numbers between 3 and 4 days, reached maximum levels at 6 days, and then rapidly declined in numbers until control levels were reached at 8 days. They were the predominant cell type from 3 to 7 days. The
FIG. 3. The effect of day-5 L(ES)-CM (10%) on the growth of neutrophils, monocytes/macrophages, and total viable cells. Other details same as for Fig. 1.

Monocyte/macrophage rapidly increased in number between 4 and 5 days, and levels were possibly still increasing at 8 days. It was the predominant cell type at 7 and 8 days. Conditioned media obtained from nonchallenged sensitized spleen cells and from challenged nonsensitized spleen cells were ineffective in supporting the growth of any cell type.

Since most of the growth was invariably due to neutrophil and monocyte/macrophage production [at the maximum (day 2), eosinophil numbers accounted for only 10% of the total], the total number of viable cells was used as an index of GM-GSF levels in subsequent experiments. A dilution analysis of the conditioned medium revealed predictable responses in both growth rates and growth potentials.

Medium conditioned for 1 day supported eosinophil production with kinetics similar to those supported by day-5 CM (Fig. 4). However, it supported no neutrophil and monocyte/macrophage production. Differential counts performed at day 2 and 4 confirmed the absence of neutrophils and monocytes/macrophages. Mouse cells (spleen and marrow) of the ICR-CD1 strain behaved in the same fashion, i.e., medium conditioned for 5 days, supported the production of neutrophils, monocytes/macrophages, and eosinophils, whereas day-1 CM supported the growth only of eosinophils. The growth kinetics of the eosinophil and neutrophil/monocyte were similar to those obtained with the C3H-HeJ strain.

Initial results of colony assay in soft agar indicated that GM-CSF was not present in day-1 CM, whereas it was in day-5 CM (Dr. Frank W. Ruscetti, personal communication). No type of growth was observed in soft agar when day-1 CM was used. Also, initial results of monocyte/macrophage CSF radioimmunoassay indicated that this factor was not present in either day-1 or day-5 CM (Dr. E. R. Stanley, personal communication).

The abrupt decline in eosinophil numbers, which began between 2 and 3 days, was investigated. Day-2 marrow medium was harvested and used to stimulate fresh marrow cells. In addition, the pelleted cells were washed and resuspended in fresh medium containing L(ES)-CM at 10%. Eosinophil production from the fresh marrow occurred to the same extent and with the same kinetics as the original marrow (Fig. 5). However, eosinophils in the original marrow preparation continued to decline at the same rate when resuspended in fresh medium. These results indicated that eosinophil depletion was not due to a depletion of Eo-GSF, nor to an inhibitor that accumulates in the marrow culture, but rather to the rapid depletion of Eo-GSF target cells.

DISCUSSION

Nonadherent spleen cells sensitized against Trichinella spiralis released GSFs when challenged in vitro with excretory–secretory products of Trichinella larvae (muscle stage). Only Eo-GSF could be detected in day-1 conditioned medium, while Eo-GSF and...
GM-GSF could be detected in day-5 conditioned medium.

This study extends the work of others and indicates that Eo-GSF is a factor distinct from GM-GSFs and that its production by sensitized lymphocytes is governed by separate control mechanisms. Furthermore, a rapid, natural way of achieving it separate from other factors is indicated.

The study also showed that, in this system or in response to these factors, the kinetics of eosinophil, neutrophil, and monocyte/macrophage production were very different. Eosinophil production occurred during the first 2 days of culture and was the only cell type being produced during this time. Eosinophil production had returned to unstimulated levels by day 4. Production of neutrophils began at 2–3 days, and they were the predominant cell type from 3 to 6 days. Monocyte/macrophage production began at 4–5 days but did not predominate until very late in the culture period (7–8 days).

The abrupt decline in eosinophil numbers was shown to be due to target cell depletion and not to Eo-GSF depletion. Eosinophil production could not be reinitiated when day-2 marrow cells were washed and resuspended with fresh conditioned medium. However, the Eo-GSF activity of the culture fluid was essentially unchanged, as shown by a test on fresh marrow cells. The rapid initiation of eosinophil production, its short duration, and its abrupt decline tempt us to speculate that the target cell of this Eo-GSF is relatively mature although, within the limits of the assay, not yet a recognizable eosinophil.

Several observations indicate that Eo-GSF is different from Eo-CSF: (A) Initial results indicate that Eo-GSF does not induce colony formation in soft agar. (B) Eo-GSF-induced growth is rapid and of short duration, whereas large colonies (some >5000 cells), which form over a 5–14-day period, are induced by Eo-CSF. (C) Eo-GSF, based on ultrafiltration, has a molecular weight less than 5000 whereas the apparent molecular weight of Eo-CSF is 50,000. Murine “eosinophilopoietin” isolated from serum of mice injected with antieosinophil serum has a molecular weight below 1300 and also stimulated maximum eosinophil production in vivo and in vitro within 2 days. (D) Eo-GSF and Eo-CSF differ in the time of release from spleen cells. In our studies, Eo-GSF was released within 24 hr. Other studies demonstrated that mouse spleen or lymph node cells incubated with either pokeweed mitogen or 2-mercaptoethanol released maximal amounts of Eo-CSF between 7 and 14 days. However, McGarry et al. reported that spleen cells from *Schistosoma mansoni* infected mice exposed to *Schistosome* egg antigens for 6–24 hr released both CSF and an “eosinophilopoiesis-stimulating factor,” which was detected in vivo. The nature of the CSF was not determined. Further studies should elucidate the relationships between these different factors.

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