Granulocyte-Macrophage Colony-Stimulating Factor Enhances the Production of Eosinophil Chemotactic Lymphokine by Egg-Associated Granulomas of <i>Schistosoma japonicum</i>-Infected Mice

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by splenic lymphocytes obtained from <i>Schistosoma japonicum</i>-infected mice was partially purified by a combination of DEAE anion-exchange chromatography, concanavalin A-Sepharose affinity chromatography, and high-pressure liquid chromatography. When this partially purified GM-CSF was added to the culture of isolated intact granulomas, eosinophil chemotactic factor (ECF) lymphokine production by granulomas was significantly enhanced. The partially purified GM-CSF also enhanced ECF lymphokine production by granuloma T cells cocultured with syngeneic macrophages and specific antigen. The partially purified GM-CSF itself had neither ECF activity nor a synergistic effect with ECF lymphokine. When normal splenic macrophages were preincubated with the partially purified GM-CSF, they potentiated the ECF production by granuloma T cells under the presence of specific antigen. Augmentation of ECF lymphokine production by partially purified GM-CSF was further confirmed by using T-cell clones that were established from granuloma T cells. These results suggest that T-cell-derived GM-CSF primarily activate macrophages so that these activated macrophages can cooperate more effectively with T lymphocytes to produce ECF. Such potentiation of macrophage-T-cell interaction by GM-CSF may be important in the mechanisms of granuloma formation during an acute stage of schistosomiasis.

Infection of mice with <i>Schistosoma japonicum</i> engendered high levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) in serum (17). When splenic lymphocytes obtained from <i>S. japonicum</i>-infected mice were cultured with soluble egg antigen (SEA) or concanavalin A (ConA), GM-CSF activity was detected in the conditioned medium (18). A time course study (17, 18) revealed that the rise in GM-CSF levels in serum or the GM-CSF-producing activity of splenic T lymphocytes coincided well with the acute stage of <i>S. japonicum</i> infection, when eosinophil-rich granulomatous lesions were rapidly formed around deposited eggs in the livers or intestines of infected hosts. Recently, we have found that eosinophil chemotactic factor (ECF) is released from isolated intact granulomas or granuloma T cells obtained from mice at the acute stage of <i>S. japonicum</i> infection (16). Granuloma T cells require specific antigen stimulation and collaboration of syngeneic macrophages to produce ECF. Because GM-CSF is known to modulate macrophage functions (11), we assumed that ECF lymphokine production by granuloma T cells might be regulated by GM-CSF. The following experiments were done to evaluate the regulatory role of GM-CSF on ECF lymphokine production by granuloma T cells.

**MATERIALS AND METHODS**

**Animals and infection.** C57BL/6 mice were raised in our animal center under clean, conventional conditions. Female mice (age, 5 to 6 weeks; weight, approximately 15 g at the time of infection) were used throughout this series of experiments. They were infected intraperitoneally with 30 cercariae of <i>S. japonicum</i> (Japanese strain).

**Antigen.** SEA was prepared by previously described methods (15).

**Preparation of partially purified GM-CSF.** Mice were sacrificed with ether and cervical dislocation 6 weeks after infection, and the spleens were removed aseptically. They were gently squashed between two frost-ended slides in cold Hanks balanced salt solution (HBSS). The cell suspension was washed with HBSS and suspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 2% heat-inactivated fetal bovine serum (FBS; Flow Laboratories, Inc., McLean, Va.) and 5 x 10⁻³ M 2-mercaptoethanol. The cells were cultured with 5 μg of SEA per ml at 37°C for 24 h in a 5% CO₂-air environment. Conditioned medium was obtained by centrifugation in 1,200 x g for 10 min. Approximately 3 liters of the conditioned medium was concentrated 60 times by ultrafiltration by using a Pericon-Labocasette System (cutoff point, 10,000 daltons; Millipore Corp., Bedford, Mass.).

(i) **Anion-exchange chromatography.** Concentrated material was dialyzed against 0.016 M Tris–hydrochloride buffer (pH 7.7) and applied onto a column (2.5 by 60 cm; DE52; Whatman, Inc., Clifton, N.J.) which was equilibrated with the same buffer. Elution was performed with a linear gradient of NaCl (0 to 0.3 M). GM-CSF activity was detected as a broad single peak at about a 0.1 M NaCl concentration.

(ii) **ConA-Sepharose 4B affinity chromatography.** GM-CSF-positive fractions from the DE52 column were pooled, concentrated by using a membrane (cutoff point, 10,000 daltons; UM-10; Amicon Corp., Lexington, Mass.), and dialyzed against ConA buffer (0.1 M acetate buffer [pH 6.0] containing 0.5 M NaCl, 1 mM CaCl₂, 1 mM MnCl₂, and 1 mM MgCl₂). The sample was applied to a ConA-Sepharose 4B (Pharmacia, Inc., Piscataway, N.J.) column (0.7 by 12.5
cm). The column was eluted first with the ConA buffer and then with the ConA buffer containing 0.2 M methyl-α-D-glucoside. More than 75% of the GM-CSF activity was detected in ConA-bound fractions.

(iii) High-pressure liquid chromatography. ConA-bound fractions containing GM-CSF were concentrated and applied to a high-pressure liquid chromatograph equipped with a column (0.75 by 70 cm; SW3000; Toyo Soda). Bovine serum albumin (molecular weight, 67,000), ovalbumin (molecular weight, 45,000), α-chymotrypsinogen A (molecular weight, 25,000), and cytochrome c (molecular weight, 13,000) were used as standards. GM-CSF activity was detected at the elution position between ovalbumin and α-chymotrypsinogen A. Thus, the apparent molecular weight of GM-CSF was calculated as 30,000.

IL-2 and interferon assay. To exclude the possibility of contamination by interleukin-2 (IL-2) or interferon in the partially purified GM-CSF, IL-2 and interferon activities in the partially purified GM-CSF were tested. IL-2 activity was examined by [3H]thymidine uptake by an IL-2-dependent cytotoxic T-cell line, which was kindly provided by M. Yoshinaga, Department of Pathology, Kumamoto University Medical School, Kumamoto, Japan. Interferon activity was determined by 50% plaque reduction assay (5) with the New Jersey strain of vesicular stomatitis virus, which was provided by T. Ohashi, Department of Microbiology, Miyasaki Medical College, with the courtesy of Y. Mimamishima. By these assay methods, neither IL-2 nor interferon activity was detected in our partially purified GM-CSF.

In vitro colony assay. Details of techniques for colony formation in soft agar have been described previously (17). Briefly, 10² bone marrow cells of normal C57BL/6 mice were plated in plastic dishes (diameter, 35 mm; Falcon 1008; Becton Dickinson Labware, Oxnard, Calif. [Div. Becton Dickinson and Co.]) in 1 ml of a mixture containing McCoy 5A medium (GIBCO), 0.3% agar (Noble agar; Difco Laboratories, Detroit, Mich.), 10% FBS (Flow Laboratories), and a 200-μl test sample which was sterilized by filtration with a Millipore filter before use. Dishes were incubated in a humidified atmosphere with 7% CO₂. Colonies were counted on day 7 of culture.

Granuloma and granuloma cell cultures. The methods for isolation of granulomas or preparation of granuloma cells followed those described by Wyler and Postlethwaite (21), with a slight modification (16) in the procedure. In brief, the liver obtained from mice that had been infected with S. japonicum for 6 weeks was suspended in 10 volumes of HBSS and homogenized gently for 15 s in a blender. Granulomas were isolated by extensive washing with cold HBSS by centrifugation at 200 × g until the supernatant became clear. To obtain granuloma cells, isolated granulomas were enzymatically digested with 500 U of collagenase (type I; Sigma Chemical Co., St. Louis, Mo.) per ml and 0.1 mg of pronase (Kaken) per ml. Dissociated cells were harvested by passage through stainless mesh (no. 100). The cells were washed three times with HBSS, and 1.5 ml of cell suspension was cultured with SEA at a cell density of 10⁵/ml in RPMI 1640 medium in a 24-well culture plate (143982; Nunc, Roskilde, Denmark). To enrich T cells, granuloma cells were passed through a nylon wool column. After enrichment, 50 to 60% of the total nucleated cells were Thy1.2-positive cells, as determined by a cytotoxicity test. A macrophage-rich cell suspension was prepared from normal spleen cells by plastic adherence. From this preparation, 3 × 10⁶ spleen cells per ml in RPMI 1640 medium containing 10% FBS were incubated in a plastic dish (8-757-12; Fisher Scientific Co., Pittsburgh, Pa.) at 37°C for 60 min. After extensive washing with the medium, adherent cells were harvested.

T-cell clones. To perform T-cell cloning, cells were cultured in RPMI 1640 medium containing bicarbonate and supplemented with L-glutamine (2 mM), pyruvate (0.1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), l-HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; 10 mM), monothioglycerol (7.5 × 10⁻⁵ M), and 10% heat-inactivated FBS. A panel of T-cell clones reactive to SEA of S. japonicum were produced by the method described by Mak and Sanderson (10), with a slight modification (18a). Briefly, T-cell-enriched granuloma cells were stimulated with 50 μg of SEA per ml in culture flasks (Falcon 3024; Becton Dickinson) for 3 days. T-cell cloning was performed by the limiting dilution method. Various numbers of lymphoblasts (0.3 to 100 per well) were cultured with 2 × 10⁵ X-ray-irradiated syngeneic spleen cells, SEA, and IL-2 in 96-well flat-bottom plates (Falcon 3072; Becton Dickinson) for 9 to 14 days. The conditioned medium obtained from ConA-stimulated spleen cells was used as the IL-2 source and was added to the culture at 20% (by volume). Proliferating cells from plates with wells that were less than 30% positive were expanded for further studies. The cells were transferred to and maintained in 24-well culture plates (Nunc) containing 10⁴ irradiated spleen cells, SEA, and IL-2. The cells were maintained by weekly restimulation with SEA. To obtain conditioned medium, T-cell clones were washed and transferred into serum-free, IL-2-free RPMI 1640 medium containing 103 splenic adherent cells per ml and 10 μg of SEA per ml. The cells were cultured for 24 h, and ECF activity in the supernatant was examined.

Eosinophils and in vitro chemotaxis. The methods for preparation of eosinophil-rich peritoneal exudate cells and those for the in vitro chemotaxis assay have been described previously (16). In brief, blind well chambers (Bio-Rad Laboratories, Richmond, Calif.) were equipped with membrane filters (pore size, 3 μm; Millipore). They were filled with 0.2 ml of eosinophil-rich cell suspension in the upper compartment and 0.2 ml of each sample in the lower compartment. The chambers were incubated for 2 h at 37°C in a 5% CO₂-in-air environment. After staining, all eosinophils which migrated between the upper and lower surfaces of the membrane were counted.

RESULTS

Effect of GM-CSF on ECF production by intact granulomas. Various concentrations of partially purified GM-CSF were added to the cultures of isolated intact granulomas. The conditioned media were harvested 24 h after culture, and their ECF activity was measured (Fig. 1). ECF activity in the conditioned medium increased proportionally with the amount of GM-CSF added to the culture.

Effect of GM-CSF on ECF production by granuloma T cells. In our previous study (16), it was revealed that granuloma T cells could, in collaboration with macrophages, produce ECF on stimulation with specific antigen. Thus, we examined whether ECF production by granuloma T cells was enhanced by partially purified GM-CSF. For this purpose, 5 × 10⁶ nylon wool-passed T-cell-enriched cells and 1 × 10⁵ syngeneic macrophages were cultured under the presence of 10 μg of SEA per ml and various concentrations of partially purified GM-CSF. The conditioned media were harvested, and the ECF activity was measured (Fig. 2). Again, ECF activity in the conditioned medium increased proportionally with the amount of GM-CSF added to the culture.
Effect of GM-CSF on ECF activity in the media conditioned by granulomas or granuloma T cells. As indicated by the results presented above, the addition of partially purified GM-CSF in the culture of isolated, intact granulomas or granuloma T cells enhanced ECF activity in the conditioned medium. Such an enhancement can be explained in two ways. One possibility is that partially purified GM-CSF added in the culture directly potentiates the ECF activity released in the medium. Alternatively, partially purified GM-CSF may act on the cells that are responsible for the production of ECF. To test these possibilities, the media conditioned by isolated intact granulomas or granuloma T cells, which were cultured without GM-CSF, were mixed with partially purified GM-CSF, and their ECF activity was examined (Table 1). ECF activity in the media conditioned by granulomas or granuloma T cells was not altered by the addition of partially purified GM-CSF. Furthermore, partially purified GM-CSF per se did not show ECF activity.

Effect of preincubation of macrophages with GM-CSF on the expression of accessory cell function. To determine whether partially purified GM-CSF acts on macrophages or on T cells, macrophages were preincubated with partially purified GM-CSF and then cocultured with granuloma T cells in the presence of SEA. ECF activity in the conditioned medium harvested from the culture of GM-CSF-treated macrophages and granuloma T cells was higher than that in the conditioned medium of untreated macrophages and granuloma T cells (Table 2).

Effect of GM-CSF on ECF production by T-cell clones. To confirm further that partially purified GM-CSF augmented ECF production by granuloma T cells, the ECF-producing activity of antigen-specific T-cell clones prepared from granuloma T cells was examined under the presence or absence of GM-CSF. When partially purified GM-CSF was added to the cultures of eight different T-cell clones under the presence of 10^6 normal syngeneic macrophages and 10 μg of SEA per ml, seven of eight clones showed significant enhancement of ECF production (Fig. 3).

### DISCUSSION

The GM-CSF used in this study was partially purified from the medium conditioned by splenic lymphocytes obtained from S. japonicum-infected mice. This GM-CSF is produced by T cells (18) and has fundamental similarities to other known T-cell-derived CSF in its physicochemical nature (3, 4, 9, 19).

The results reported here indicate that partially purified GM-CSF enhances ECF production by isolated intact granulomas.

### TABLE 1. Effect of GM-CSF on ECF activity in the media conditioned by isolated intact granulomas or granuloma T cells

<table>
<thead>
<tr>
<th>Media conditioned by:</th>
<th>Chemotaxis (no. of eosinophils/10 hpf)</th>
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<tbody>
<tr>
<td></td>
<td>CSF^-</td>
</tr>
<tr>
<td>Granulomas</td>
<td>95 ± 11</td>
</tr>
<tr>
<td>Granuloma T cells</td>
<td>89 ± 3</td>
</tr>
<tr>
<td>Phosphate-buffered saline</td>
<td>0</td>
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</tbody>
</table>

* The media conditioned by isolated intact granulomas or granuloma T cells were prepared as described in the text without the addition of CSF. Partially purified CSF (80 U) was added to the conditioned medium, and ECF activity was measured.

### TABLE 2. Effect of preincubation of macrophages with GM-CSF on the expression of accessory cell function

<table>
<thead>
<tr>
<th>Macrophage preincubation with CSF (80 U)</th>
<th>Incubation with:</th>
<th>T cell</th>
<th>CSF (80 U)</th>
<th>Chemotaxis (no. of eosinophils/10 hpf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>135 ± 10</td>
<td></td>
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<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>193 ± 8</td>
<td></td>
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<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>64 ± 2</td>
<td></td>
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<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>0</td>
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</table>

* A total of 10^6 splenic macrophages were preincubated with or without partially purified GM-CSF for 24 h. After preincubation, macrophages were washed and then cocultured with 5 x 10⁶ granuloma T cells in the presence of SEA (10 μg/ml) for 24 h. ECF activity in the supernatant was examined. The background count of eosinophil migration toward the conditioned media prepared in the absence of antigen was less than 10 of 10 hpf.
ulomas (Fig. 1), by enzymatically dispersed granuloma T cells (Fig. 2), or by cloned T cells (Fig. 3). When splenic macrophages were preincubated with partially purified GM-CSF, their accessory function on ECF production by granuloma T cells was significantly enhanced (Table 2). Recently, GM-CSF was considered to be not only a myelopoeitin but to have a wider range of regulatory roles on the function of macrophages and neutrophils (11). For example, GM-CSF stimulates macrophages to produce interleukin-1 (12), interferon (13), and plasminogen activator (7). Results of this study provide good evidence that GM-CSF enhances accessory function of macrophages in lymphokine production. Grabstein et al. (6) have reported that GM-CSF acts on splenic macrophages to activate an accessory function for B lymphocytes in antigen-specific antibody responses, which is similar to the results of this study.

In schistosomiasis, eosinophil-rich granulomas are formed around deposited eggs (20). Accumulation of eosinophils and other cell types around schistosome eggs seems to be mediated, at least in part, by both parasite-derived and host-derived chemoattractants. As the parasite-derived chemoattractants, ECF (15) and neutrophil chemotactic factor (14) have been isolated from S. japonicum eggs. Apart from the parasite-derived factors, various types of host-derived factors, most of which are assumed to be lymphokines or cytokines, have been reported. For example, we have reported that granuloma T cells produce ECF (16). Fibroblast chemotactic factor, which is indistinguishable from fibronectin, is produced from granuloma macrophages of S. mansoni-infected mice (21). Furthermore, fibroblast-stimulating factor is produced by granuloma macrophages (22) or T-cell clones (8) of S. mansoni-infected mice. From these previous observations, there is little doubt about the importance of macrophages and T cells as the regulators of the formation of granulomatous lesions around deposited eggs in schistosomiasis. Indeed, the possible importance of T cells in granulomatous response in S. japonicum-infected mice has been reported with athymic (2) or B-cell-depleted (1) mice. In this study, partially purified GM-CSF, which is produced by T lymphocytes under the presence of macrophages and specific antigen (16), could stimulate the accessory function of macrophages so that these activated macrophages could collaborate more effectively with T lymphocytes to produce ECF lymphokine. Such an augmentation of the mutual interaction of T cells and macrophages by GM-CSF is one of the important mechanisms of eosinophil-rich granuloma formation in S. japonicum infection. As mentioned above, GM-CSF is known to have a broad range of regulatory functions on macrophages (11). Thus, there is a possibility that not only a macrophage-dependent T-cell-eosinophil system but also other macrophage-dependent cellular interaction systems, such as a T-cell-fibroblast system or a macrophage-fibroblast system are under the regulation of GM-CSF. Such possibilities should be explored in the future.

LITERATURE CITED


FIG. 3. Effect of GM-CSF on ECF production by T-cell clones. Purified GM-CSF was added to the cultures of eight different T-cell clones under the presence of 10⁶ normal syngeneic macrophages and 10 μg of SEA per ml. The conditioned media were harvested, and their ECF activity was examined. ECF activity in the absence of antigen was not measured because the growth of these T-cell clones was dependent on the presence of specific antigen. Horizontal bars represent the standard error of the mean. Symbols: *, P < 0.05; **, P < 0.01. Eos., Eosinophil.


