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Granulocyte-Macrophage Colony-Stimulating Factor in the Sera of *Schistosoma japonicum*-Infected Mice

MAKOTO OWHASHI AND YUKIFUMI NAWA*

Department of Parasitology, Miyazaki Medical College, Kiyotake, Miyazaki 889-16, Japan

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Infection of mice with *Schistosoma japonicum* engendered high levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) in serum. The rise in GM-CSF levels in serum was closely associated with the acute phase of the infection and seemed to be dependent on the dose of infection. GM-CSF activity was detected as a sharp single peak in DE-52 anion-exchange chromatography and Sephacryl S-200 and Sephadex G-200 gel chromatography and was almost entirely adsorbed to concanavalin A-Sepharose 4B affinity chromatography. The possible immunological and immunopathological importance of GM-CSF in *S. japonicum* infection is discussed.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is required for the proliferation and differentiation of normal granulocyte-macrophage precursor cells in vitro (3). Recent findings, however, suggest that colony-stimulating factor (CSF) may play a larger role in regulating the macrophage-mononuclear phagocyte system than does a myelopoietin (14). For example, CSF stimulates macrophages to produce interleukin 1 (15), as well as prostaglandins (11), plasminogen activator (7), and interferon (16), which are all humoral factors involved in the regulation of inflammatory reactions and immune responses.

In *Schistosoma japonicum* infection, both macrophages and neutrophils, together with eosinophils, play an important role not only in the formation of granulomatous lesions but also in the schistosomula-killing mechanism (21). Furthermore, a rise in CSF levels in serum has been reported in various bacterial (24) and viral (6) infections. Thus, there is a possibility that GM-CSF is produced during *S. japonicum* infection and acts as one of the regulators of inflammatory reactions or immune responses or both. In the present study, GM-CSF levels in the serum of *S. japonicum*-infected mice were examined, and some physicochemical characterizations of serum GM-CSF were performed.

MATERIALS AND METHODS

Serum preparation from *S. japonicum*-infected mice. BALB/c mice were infected with *S. japonicum* (Kofu strain) by intraperitoneal injection with 30 cercariae unless otherwise stated. Sera obtained from infected mice were pooled and stored at −30°C until used. Pooled sera taken from mice 8 weeks after infection were used for physicochemical analysis of CSF.

Preparation of SjAW-ext, excretory-secretory products, soluble egg antigen, and antiserum against SjAW-ext. Methods for the preparation of adult worm extract (SjAW-ext), excretory-secretory products, and soluble egg antigen have been described elsewhere (8, 20). Antiserum against SjAW-ext was prepared in rabbits. This antiserum showed strong precipitin lines not only against SjAW-ext and excretory-secretory products but also against heavily infected mouse serum, suggesting its ability to detect circulating antigen.

In vitro colony assay. Details of techniques for colony formation in soft agar have been described previously (T. Hayama, Y. Nawa, and M. Kotani, Exp. Hematol. [Lawrence], in press). Briefly, 10^5 bone marrow cells of normal C57BL/6 mice were plated in 35-mm plastic dishes (Falcon 1008; Becton Dickinson Labware) in 1 ml of a mixture containing RPMI 1640 medium (GIBCO Laboratories), 0.3% agar (Noble agar; DIFCO), 10% fetal calf serum (GIBCO), and a 200-μl test sample which was sterilized by Millipore filtration (Millipore Corp.) before use. Dishes were incubated in a humidified atmosphere with 7% CO_2. Colonies were counted on day 7 of culture. For differential counting of colonies, agar cultures were dried on glass slides, stained by β-glucuronidase reaction, and counterstained with hematoxylin (Hayama et al., in press). Colonies were classified as enzyme reaction-negative granulocyte colonies, enzyme reaction-positive macrophage colonies, or a mixture of both. All assays were performed as duplicate cultures and repeated at least twice.

Anion-exchange chromatography. A DE-52 column (1.6 by 34.5 cm; Whatman, Inc.) was equilibrated with 0.017 M Tris hydrochloride buffer (pH 7.7) containing 0.05% polyethylene glycol. Elution was carried out at a flow rate of 100 ml/h at 4°C, and a 9.3-ml fraction was collected. The column was washed with the equilibrating buffer, and then bound material was eluted with a linear-gradient increase of NaCl (0 to 0.3 M). The column was finally washed with 0.5 M NaCl.

Gel filtration. Sephacryl S-200 (Pharmacia, Inc.) was prepared as a column (2.5 by 95 cm) and equilibrated with 5.8 mM phosphate-buffered saline (pH 7.4) containing 0.05% polyethylene glycol. Elution was carried out with the same buffer at a flow rate of 21 ml/h at 4°C, and a 9.3-ml fraction was collected. Blue dextran (Pharmacia), ovalbumin (Sigma Chemical Co.), cytochrome *c* (Sigma), and NaCl were used as molecular weight markers for the Sephacryl S-200 column. A Sephadex G-200 column (1.6 by 65 cm) was equilibrated with the same buffer. Elution was carried out at a flow rate of 4 ml/h at 4°C, and a 2.2-ml fraction was collected. Blue dextran, ferritin (Boehringer Mannheim Biochemicals), catalase (Sigma), aldolase (Sigma), bovine serum albumin

* Corresponding author.

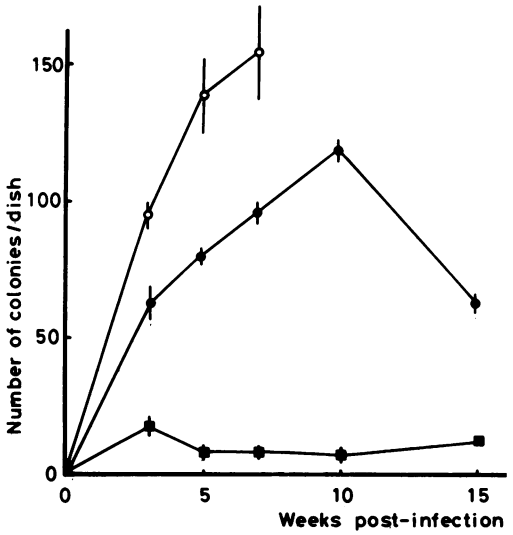

FIG. 1. GM-CSF levels in sera of BALB/c mice at various times after infection with 10 (■), 30 (●), or 100 (○) cercariae of *S. japonicum*. The mean worm recovery rates at the end of the experiment (15 weeks for the groups infected with 10 and 30 cercariae and 7 weeks for the groups infected with 100 cercariae) were 3 ± 1, 10 ± 4, and 36 ± 2, respectively. Vertical bars, Standard deviation of the mean.

(Sigma), and ovalbumin were used as molecular weight markers for the Sephadex G-200 column.

Affinity chromatography on ConA-Sepharose 4B. A concanavalin A (ConA)-Sepharose 4B column (Pharmacia; 0.7 by 12.5 cm) was equilibrated with ConA buffer (0.1 M acetate buffer [pH 6.0] containing 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$). Elution was carried out at a speed of 3.2 ml/h at 4°C, and a 2.0-ml fraction was collected. Bound material was subsequently eluted with ConA buffer containing 0.2 M methyl-α-D-glucoside.

TABLE 1. Differential count of colonies generated by *S. japonicum*-infected mouse serum

No. of cercariae used for infection	No. of colonies[a]	Percentage of colonies identified as:[b]		
		Granalocytes	Macrophages	Mixed
30	152 ± 16	18.4	61.8	19.7
100	199 ± 18	22.1	58.3	19.6

[a] Mean ± standard deviation of two separate duplicate cultures.
[b] Colonies were typed by enzyme reaction.

RESULTS

Time course study. Pooled sera (20 mice per pool for each dose level) obtained at various time intervals after infection with various doses of *S. japonicum* cercariae were diluted five times with phosphate-buffered saline to measure colony-stimulating activity. Colony-stimulating activity became detectable at 3 weeks after infection (Fig. 1). At this time, activity seemed to be dependent on the initial level of infection. When mice were infected with 30 or 100 cercariae, colony-stimulating activity increased further with time, whereas colony-stimulating activity in mice given 10 cercariae decreased nearly to the levels of uninfected controls. In the group infected with 30 cercariae, peak CSF activity was observed at 10 weeks after infection, and then CSF activity gradually decreased. In the group infected with 100 cercariae, a majority of the mice died by 8 weeks after infection.

Relative CSF concentrations and colony morphology. The relative concentrations of CSF in sera obtained 7 weeks after moderate (30 cercariae) or heavy (100 cercariae) infection were determined by titration curves (Fig. 2). According to these data, CSF activity in heavily infected mouse serum was approximately twofold higher than that in moderately infected mouse serum. Morphological analysis (Table 1) revealed that the colonies generated by these two samples were granulocytes, macrophages, or a mixture of both cell types.

Anion-exchange chromatography. Pooled mouse serum (25 ml) obtained at 8 weeks after infection with 30 cercariae was applied to a DE-52 column. The elution pattern is shown in

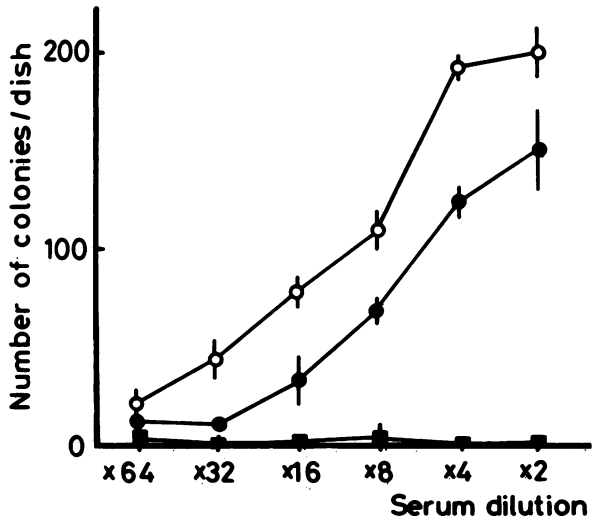

FIG. 2. Titration curve of GM-CSF activity in mouse sera obtained 7 weeks after infection with 30 (●) or 100 (○) *S. japonicum* cercariae. Serially diluted normal mouse serum (■) was used as a negative control. Vertical bars, Standard deviation of the mean.

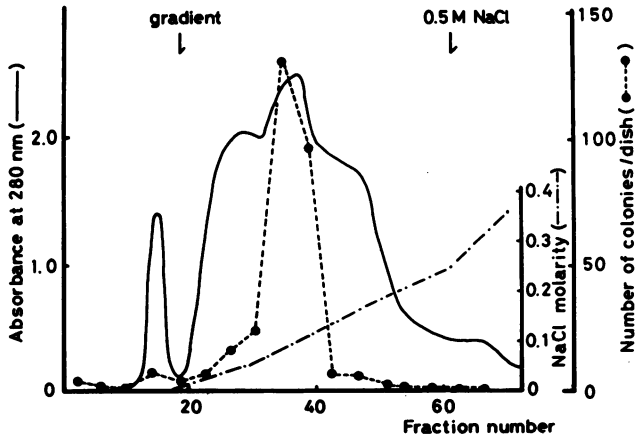

FIG. 3. DE-52 anion-exchange chromatography of mouse serum obtained 8 weeks after infection with 30 *S. japonicum* cercariae. GM-CSF activity is expressed as the number of colonies generated per dish (●). The optical density at 280 nm (—) and NaCl molarity (-·-·-) of each fraction are also shown.

Fig. 3. Groups of four fractions each were pooled. CSF activity was detected as a single peak eluted at approximately the 0.1 M NaCl region of the gradient.

Gel chromatography. CSF-positive fractions from the DE-52 column were pooled, concentrated by UM-10 ultrafiltration (Amicon Corp.), and applied to a Sephacryl S-200 column. The elution pattern is shown in Fig. 4. The major peak of CSF activity was detected at the void volume. The peak fractions were pooled and rechromatographed on a Sephadex G-200 column. The elution pattern is shown in Fig. 5. CSF activity was detected as a single peak slightly bigger than that of catalase. From this result, the molecular weight of CSF in serum was estimated as 260,000.

Affinity chromatography. Since many previously reported CSFs can bind to ConA-Sepharose 4B (2), the affinity of Sephadex G-200 gel-chromatographed CSF to ConA was tested. CSF was almost entirely adsorbed onto ConA-Sepharose 4B and was eluted by methyl-α-D-glucoside (Fig. 6).

CSF activity in parasite-derived components. There is a possibility that such a high-molecular-weight, biologically active substance is derived from parasites, because its timing and response to GM-CSF parallel data obtained on circulating antigens of a polysaccharide or glycoprotein nature (4, 17, 22). Thus, CSF activities in SjAW-ext, excretory-secretory products, and soluble egg antigen were examined. The results show that the mean numbers of colonies generated by these parasite-derived components were less than five per dish, which was within the background level of unstimulated culture. Furthermore, when partially purified GM-CSF (after the Sephadex G-200 step [see above]) was tested against rabbit anti-SjAW-ext serum by double diffusion in agar gel, a precipitin line was not detected.

DISCUSSION

The results reported here show that *S. japonicum* infection in mice caused a rapid and relatively persistent rise in GM-CSF levels in serum. Such an elevation of CSF levels in serum has already been reported after viral or bacterial infections (6, 24). Thus, not only viral or bacterial infections, but also parasitic infection, can elicit an elevation of CSF levels in serum.

There is a possibility that a parasite-derived component in the circulatory system, such as circulating antigen (4, 17, 22), has CSF activity per se. This possibility is, however, rather unlikely because, as far as we have determined, CSF activity was not present in various parasite-derived components such as SjAW-ext, excretory-secretory products, and soluble egg antigen.

Concerning the mechanism of production of GM-CSF, at least two main possibilities should be considered. One is increased demand owing to granulopenia, and the other is direct stimulation of CSF sources, such as macrophages or fibroblasts or both, by parasite-derived components. In the present study, elevation of CSF levels in serum was observed during an acute phase of *S. japonicum* infection when the granulocyte count in peripheral blood increased (data not shown). Therefore, elevation of CSF in serum seems to be the result of direct stimulation of CSF sources by *S.*

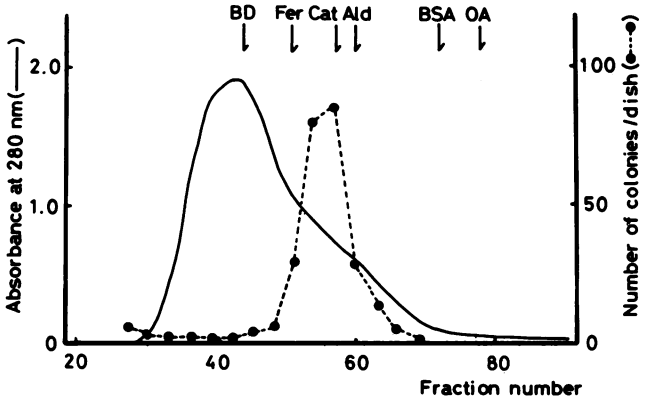

FIG. 5. Sephadex G-200 gel chromatography of the GM-CSF-positive fractions from Sephacryl S-200 gel chromatography. GM-CSF activity is expressed as the number of colonies generated per dish. Blue dextran (BD), ferritin (Fer), catalase (Cat), aldolase (Ald), bovine serum albumin (BSA), and ovalbumin (OA) were eluted at the positions indicated at the top of the figure.

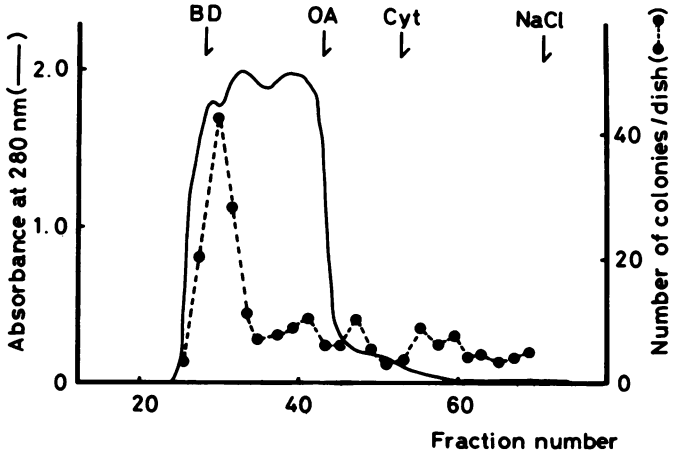

FIG. 4. Sephacryl S-200 gel chromatography of the GM-CSF-positive fractions from DE-52 anion-exchange chromatography. GM-CSF activity is expressed as the number of colonies generated per dish. Blue dextran (BD), ovalbumin (OA), cytochrome *c* (Cyt), and NaCl were eluted at the positions indicated at the top of the figure.

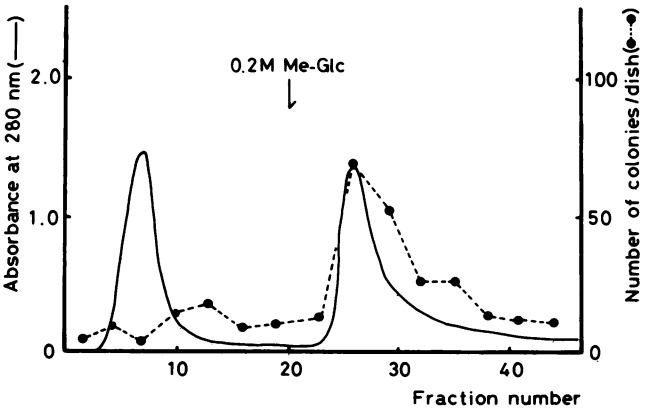

FIG. 6. ConA-Sepharose 4B affinity chromatography of the GM-CSF-positive fractions from Sephadex G-200 gel chromatography. GM-CSF activity is expressed as the number of colonies generated per dish. Bound material was eluted by ConA buffer containing 0.2 M methyl-α-D-glucoside (Me-Glc).

japonicum worms or eggs or both. In the life cycle of *S. japonicum*, egg deposition begins at approximately 5 weeks after skin penetration (21). In the present study, CSF became detectable at 3 weeks after infection and rapidly rose up to 10 weeks after infection. Thus, both immature adult worms (schistosomula) and mature adult worms or eggs or both seem to be causative stimuli for CSF production.

GM-CSF reported here was detected as a sharp, single peak in DE-52 anion-exchange chromatography (Fig. 3), Sephacryl S-200 (Fig. 4) and Sephadex G-200 (Fig. 5) gel chromatography, and ConA-Sepharose affinity chromatography (Fig. 6). Such physicochemical homogeneity is rather unusual compared with the heterogenous nature of previously reported CSFs from various sources on anion-exchange chromatography (19) or ConA affinity chromatography (26). In terms of the physicochemical nature of CSF, the apparent molecular weight of CSF in *S. japonicum*-infected mouse serum was 260,000 as measured by Sephadex G-200 gel chromatography. Furthermore, the activity was almost entirely adsorbed by ConA-Sepharose 4B and eluted by methyl-α-D-glucoside, suggesting a glycoprotein nature. An anomalously high apparent molecular weight of GM-CSFs estimated by gel chromatography has been reported in murine lung CSF (18), L-cell CSF (26), human urinary CSF (23), and CSF from estriol-treated mouse serum (Hayama et al., in press) and was attributed to the probable glycoprotein nature of CSF.

As to the functional importance of granulocytes and macrophages in schistosome infection, Maeda et al. (13) reported neutrophil-rich response to schistosomula in the skin of immunized mice. Furthermore, neutrophil-mediated, antibody-dependent (9) and lymphokine-activated, macrophage-mediated schistosomula killing (1, 10) has been reported. Since neutrophil-mediated, antibody-dependent cytotoxicity is enhanced by GM-CSF (12, 25), and an array of macrophage functions are altered by GM-CSF (14), these forms of cell-mediated schistosomula killing may be augmented by GM-CSF. Related to this, Dessein et al. (5) reported that semipurified eosinophil colony-stimulating factor could enhance eosinophil cytotoxicity against schistosomula. In addition to schistosomula killing, macrophages and neutrophils, as well as eosinophils, play an important role in the formation and modification of egg-associated granulomatous lesions (21). Therefore, it is reasonable to postulate that GM-CSF, in one way or another, contributes to the mechanism of granuloma formation. Biological functions, other than that of a myelopoietin, of CSF obtained from *Schistosoma*-infected mouse serum should be further clarified.

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LITERATURE CITED

1. **Bout, D. T., M. Joseph, J. R. David, and A. R. Capron.** 1981. In vitro killing of *S. mansoni* schistosomula by lymphokine-activated mouse macrophages. J. Immunol. **127**:1–5.
2. **Burgess, A. W., and D. Metcalf.** 1980. The nature and action of granulocyte-macrophage colony stimulating factors. Blood **56**:947–958.
3. **Bradley, T. R., and D. Metcalf.** 1966. The growth of mouse bone marrow cells in vitro. Aust. J. Exp. Biol. Med. Sci. **44**:287–300.
4. **Carlier, Y., D. Bout, G. Strecker, H. Debray, and A. Capron.** 1980. Purification, immunochemical and biologic characterization of the *Schistosoma* circulating M antigen. J. Immunol. **124**:2442–2450.
5. **Dessein, A. J., M. A. Vadas, N. A. Nicola, D. Metcalf, and J. R. Devid.** 1982. Enhancement of human blood eosinophil cytotoxicity by semi-purified eosinophil colony stimulating factor(s). J. Exp. Med. **156**:90–103.
6. **Foster, R., D. Metcalf, and R. Kirchmyer.** 1968. Induction of bone marrow colony stimulating activity by a filterable agent in leukemic and normal mouse serum. J. Exp. Med. **127**:853–866.
7. **Hamilton, J. A., E. R. Stanley, A. W. Burgess, and R. K. Schadduck.** 1980. Stimulation of macrophage plasminogen activator activity by colony-stimulating factors. J. Cell. Physiol. **103**:435–445.
8. **Horii, Y., M. Owhashi, A. Ishii, K. Bandou, and M. Usui.** 1984. Eosinophil and neutrophil chemotactic activities of adult worm extracts of *Schistosoma japonicum* in vivo and in vitro. J. Parasitol. **70**:955–961.
9. **Incani, R. N., and D. J. McLaren.** 1981. Neutrophil-mediated cytotoxicity to *Schistosoma mansoni* in vitro: studies on the kinetics of complement and/or antibody-dependent adherence and killing. Parasite Immunol. **3**:107–126.
10. **James, S. L., J. K. Lazdins, S. Hieny, and P. Natvitz.** 1983. Macrophages as effector cells of protective immunity in murine schistosomiasis. VI. T cell-dependent, lymphokine-mediated activation of macrophages in response to *Schistosoma mansoni* antigens. J. Immunol. **131**:1481–1486.
11. **Kurland, J. I., L. M. Pelus, P. Ralph, R. S. Bockman, and M. A. S. Moore.** 1979. Induction of prostaglandin E synthesis in normal and neoplastic macrophages: role for colony-stimulating factor(s) distinct from effects on myeloid progenitor cell proliferation. Proc. Natl. Acad. Sci. U.S.A. **76**:2326–2330.
12. **Lopez, A. F., N. A. Nicola, A. W. Burgess, D. Metcalf, F. L. Battye, W. A. Sewell, and M. A. Vadas.** 1983. Activation of granulocyte cytotoxic function by purified mouse colony-stimulating factors. J. Immunol. **131**:2983–2988.
13. **Maeda, S., Y. Irie, and K. Yasuraoka.** 1982. Resistance of mice to secondary infection with *Schistosoma japonicum*, with special reference to neutrophil enriched response to schistosomula in the skin of immune mice. Jpn. J. Exp. Med. **52**:111–118.
14. **Moore, R. N., J. T. Hoffeld, J. J. Farrar, S. E. Mergenhagen, J. J. Oppenheim, and R. K. Shadduck.** 1981. Role of colony-stimulating factors as primary regulators of macrophage functions. Lymphokines **3**:119–148.
15. **Moore, R. N., J. J. Oppenheim, J. J. Farrar, C. S. Carter, Jr., A. Waheed, and R. K. Shadduck.** 1980. Production of lymphocyte-activating factor (interleukin 1) by macrophages activated with colony-stimulating factors. J. Immunol. **125**:1302–1305.
16. **Moore, R. N., S. N. Vogel, L. M. Wahl, and S. E. Mergenhagen.** 1980. Factors influencing lipopolysaccharide-induced interferon production, p. 131–134. *In* D. Schlessinger (ed.), Microbiology—1980. American Society for Microbiology, Washington, D.C.
17. **Nash, T. E., B. Prescott, and F. A. Neva.** 1974. The characteristics of a circulating antigen in schistosomiasis. J. Immunol. **112**:1500–1507.
18. **Nicola, N. A., A. W. Burgess, and D. Metcalf.** 1979. Similar molecular properties of granulocyte-macrophage colony-stimulating factors produced by different mouse organs in vitro and in vivo. J. Biol. Chem. **254**:5290–5299.
19. **Ohno, T., M. Seki, and M. Shikita.** 1978. Colony stimulating factors on human bone marrow cells from Yoshida sarcoma cell line. Blood **51**:911–918.
20. **Owhashi, M., and A. Ishii.** 1982. Purification and characterization of a high molecular weight eosinophil chemotactic factor from *Schistosoma japonicum* eggs. J. Immunol. **129**:2226–2231.
21. **Phillips, S. M., and D. G. Colley.** 1978. Immunologic aspects of host responses to schistosomiasis: resistance, immunopathology, and eosinophil involvement. Prog. Allergy **24**:49–182.
22. **Qian, Z. L., and A. M. Deelder.** 1983. Schistosoma japonicum: immunological response to circulating polysaccharide antigens in rabbits with a light infection. Exp. Parasitol. **55**:394–403.

23. **Stanley, E. R., and D. Metcalf.** 1971. The molecular weight of colony stimulating factor (CSF). Proc. Soc. Exp. Biol. Med. **137**:1029–1031.
24. **Trudgett, A., T. A. McNeill, and M. Killen.** 1973. Granulocyte-macrophage precursor cell and colony-stimulating factor responses of mice infected with *Salmonella typhimurium*. Infect. Immun. **8**:450–455.
25. **Vadas, M. A., N. A. Nicola, and D. Metcalf.** 1983. Activation of antibody-dependent cell-mediated cytotoxicity of human neutrophils and eosinophils by separate colony-stimulating factors. J. Immunol. **130**:795–799.
26. **Waheed, A., and R. K. Shadduck.** 1979. Purification and properties of L cell-derived colony stimulating factor. J. Lab. Clin. Med. **94**:180–194.