TEMPORARY APPEARANCE OF A CIRCULATING GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR IN LETHAL MURINE MALARIA

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Abstract. Infection of mice with *Plasmodium berghei* engendered a temporary appearance of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the serum. The peak of GM-CSF levels was detected at day 2 post-infection, and then gradually decreased. On the other hand, the number of committed stem cells for granulocytes and macrophages (CFU-GM) in bone marrow transiently decreased at day 2 post-infection, and then increased and peaked at day 6 post-infection. When the serum of *P. berghei*-infected mice was fractionated by gel chromatography on Sephacryl S-300, GM-CSF activity was detected as a single peak with an apparent molecular weight of 64 KDa. GM-CSF was entirely adsorbed to concanavalin A-Sepharose 4B affinity chromatography, and was sensitive to pronase digestion, indicating its glycoprotein nature. These results suggest that the circulating GM-CSF would contribute the increase of granulocyte-macrophage hemopoiesis in the early phase of malaria.

INTRODUCTION

The differentiation and proliferation of hemopoietic cells is regulated by a series of colony stimulating factors (CSFs). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is required for the differentiation and maturation of cell precursors in the bone marrow into granulocytes and macrophages (Bradley and Metcalf, 1966). In addition, recent findings suggest that GM-CSF also has a role for enhancing neutrophil or macrophage phagocyte system (Moore *et al*, 1981; Fleischmann *et al*, 1986); a key function of these cells in host defense. Furthermore, GM-CSF enhances the accessory activity of dendritic cells (Bowers *et al*, 1990), or stimulates macrophages to produce interleukin 1 (Moore *et al*, 1980a), interferon (Moore *et al*, 1980b) or eosinophil chemotactic factor (Owhashi *et al*, 1987), a humoral factor involved in the regulation of inflammatory reaction and/or immune response.

In malaria, we previously showed the remarkable increase in number of hemopoietic stem cells in bone marrow or in spleen (Asami *et al*, 1992), indicating the increased demand of hemopoiesis. On the other hand, Waki *et al* (1993) showed that the administration of recombinant G-CSF resulted in a protective effect against nonlethal murine malaria. These facts indicate the potential importance of granulocyte-macrophage hemopoiesis for self-defense mechanisms in malaria. In the present study, therefore, we examined GM-CSF in the serum of *Plasmodium berghei*-infected mice.

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MATERIALS AND METHODS

Serum preparation from *P. berghei*-infected mice

C57 BL/6 mice were infected with *P. berghei* (NK65) by intraperitoneal injection with 1×10^6 parasitized erythrocytes. Sera obtained from 10 mice were pooled and stored at -20°C until used.

In vitro colony assay

Details of techniques for colony formation in soft agar have been described previously (Owhashi and Nawa, 1985). Briefly, 10^5 bone marrow cells of normal or *P. berghei*-infected C57BL/6 mice were plated in 35 mm plastic dishes (Falcon 1008; Becton Dickinson Labware, Oxnard, CA, USA) in 1 ml of a mixture containing McCoys 5A medium (Gibco

BRL, Gland Island, NY, USA), 0.3% agar (Noble agar; Difco), 10% fetal calf serum (Gibco BRL), and a test sample or a culture supernatant of L929 cells (200 µl) which was sterilized by Millipore filtration (Millipore Co, Bedford, MA, USA) before use. Dishes were incubated in a humidified atmosphere with 7% CO_2. Colonies were counted on day 7 of the culture. GM-CSF activity was expressed as the number of colonies generated per dish (mean ± SD).

Gel chromatography on Sephacryl S-300

Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden) was prepared as a column (2.5 by 95 cm) and equilibrated with 5.8 mM PBS (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 9.3 ml fractions were collected. Blue dextran (Pharmacia), ferritin (Boehringer Mannheim, Germany), BSA (Sigma Chemical Co, St Louis, MO, USA), ovalbumin (Sigma), cytochrome c (Sigma) and NaCl were used as molecular weight markers for the Sephacryl S-300 column.

Affinity chromatography on Con A-Sepharose 4B

A concanavalin A (Con A)-Sepharose 4B column (Pharmacia) with bed dimensions of 0.7 by 12.5 cm was equilibrated with Con A 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/hour at 4°C, and 2.0 ml fractions were collected. Bound materials were subsequently eluted with Con A buffer containing 0.2 M methyl-α-D-glucoside.

Heating or pronase treatment

Heating was carried out at 56°C for 30 minutes in a water bath. Treatment with pronase (0.1 mg/ml) (Kaken, Tokyo, Japan) was carried out at 37°C for 1 hour with continuous shaking.

RESULTS

Kinetics of the granulocyte-macrophage hemopoiesis of *P. berghei*-infected mice were examined using *in vivo* colony assays. When normal mouse serum was examined for GM-CSF, no detectable activity was observed. On the other hand, significant levels of GM-CSF were observed in the early phase of *P. berghei*-infected mice. The peak of GM-CSF level was detected at day 2 post-infection, and then gradually decreased (Fig 1A). In bone marrow of *P. berghei*-infected mice, the number of committed stem cells for granulocytes and macrophages (CFU-GM) slightly decreased at day 2 post-infection and then increased. The number of CFU-GM increased up to 1.8-fold over the normal level on day 6, and then rapidly decreased to less than 1/5 of the normal level on day 14.

To study the physicochemical properties of the serum-derived GM-CSF, 2.0 ml of pooled mouse serum obtained at day 2 post-infection was applied to a Sephacryl S-300 column. The elution pattern and GM-GSF activity of each fraction are shown in Fig 2A. GM-CSF activity was detected as a single peak eluted at the position between BSA and ovalbumin. The molecular weight of GM-CSF was estimated as 64 kDa. Since many previously reported CSFs can bind to Con A-Sepharose 4B (Burgess and Metcalf, 1980), the affinity of the serum-derived GM-CSF to Con A was tested. Five hundred microliter of infected mouse serum was applied on Con A-Sepharose 4B, and eluted with a competitive sugar: methyl-α-D-glucoside. GM-CSF was almost adsorbed on to Con A-Sepharose 4B and was eluted by methyl-α-D-glucoside, indicating its glycoprotein nature. When the serum of *P. berghei*-infected mice was heated at 56°C for 30 minutes, GM-CSF activity was about 80% of the untreated control. On the other hand, the GM-CSF activity was completely lost by pronase digestion.

DISCUSSION

GM-CSF is a potent hematopoietic growth factor which exerts its effects on hematopoietic cell growth both *in vivo* and *in vitro*. The results reported here show that *P. berghei*-infection in mice caused a rapid and transient increase in GM-CSF levels in serum. Such an elevation of CSF levels in serum has already been reported after viral or bacterial infections (Foster *et al*, 1968, Trudgett *et al*, 1973) or in murine malaria (Mungyer *et al*, 1983). In addition, we showed that *S. japonicum*-infection also cause elevation of GM-CSF level in the serum

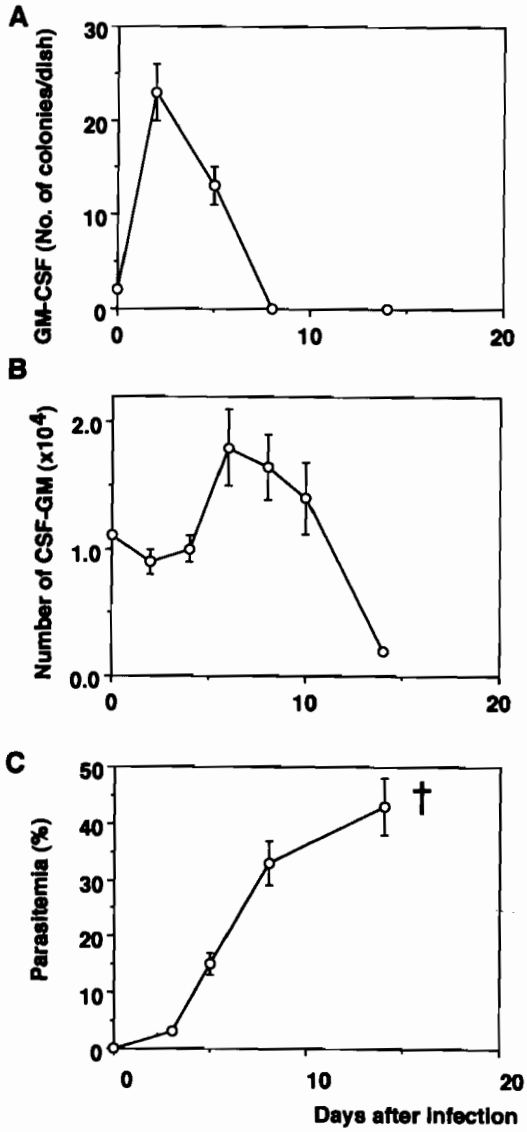

Fig 1–Time course study on hemopoiesis of *P. berghei*-infected mice. A. Kinetic study of GM-CSF level in the sera of *P. berghei*-infected mice. Pooled sera from 10 mice obtained at various time intervals after the infection were diluted 5 times with PBS to measure colony-stimulating activity. 10^5 bone marrow cells from normal mice were used for *in vitro* colony assay. GM-CSF activity is expressed as the number of colonies generated per dish (mean ± SD). B. Kinetic study of CFU-GM level in the bone marrow of *P. berghei*-infected mice. The number of colonies formed with bone marrow cells from *P. berghei*-infected mice in the presence of the supernatant of L929 cells was counted. CFU-GM is expressed as the calculated total number of GM colonies generated per femur (mean ± SD). C. Kinetic change of parasitemia. Each point represents the mean ± SD of 5 mice.

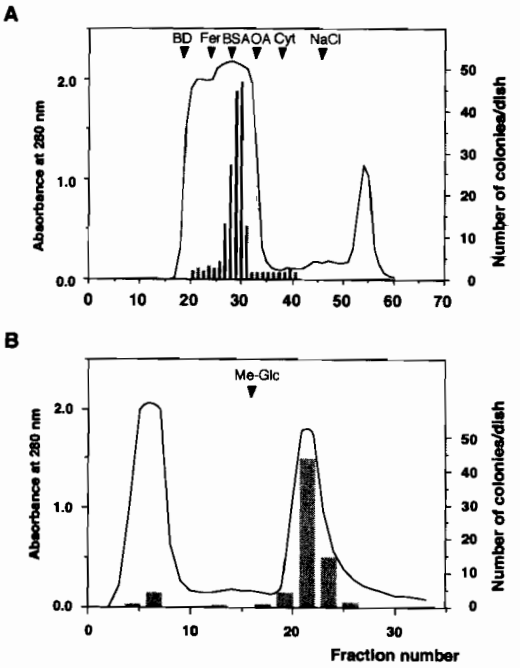

Fig 2–Analysis of the serum-derived GM-CSF. A. Sephacryl S-300 gel chromatography of *P. berghei*-infected mouse serum. Absorbance at 280 nm (▬) or GM-CSF activity (▩) of each fraction is shown. GM-CSF activity is expressed as the mean number of colonies generated per dish. Blue dextran (BD), ferritin (Fer), BSA, ovalbumin (OA), cytochrome c (Cyt) and NaCl were used for molecular weight standards. B. Con A-Sepharose 4B affinity chromatography of *P. berghei*-infected mouse serum. Absorbance at 280 nm (▬) or GM-CSF activity (▩) of each fraction is shown. Bound materials eluted by Con A buffer containing 0.2 M methyl-α-D-glucoside (Me-Glc). The activity of GM-CSF is expressed as the mean number of colonies generated per dish.

(Owhashi and Nawa 1985). Thus, not only viral, bacterial or helminthous parasite-infections but also protozoan infection, can elicit an elevation of CSF levels in serum.

GM-CSF reported here was detected as a single peak in Sephacryl S-300 gel chromatography (Fig 2A), and Con A-Sepharose affinity chromatography (Fig 2B). Such physicochemical homogeneity is rather unusual when compared with the heterogeneous nature of previously reported CSFs from

various sources on anion-exchange chromatography (Ohno *et al*, 1978) or Con A Sepharose affinity chromatography (Waheed and Shadduck, 1979). In terms of the physicochemical nature of CSF, the apparent molecular weight of GM-CSF in *P. berghei*-infected mouse serum was 64,000 as measured by Sephacryl S-300 gel chromatography. This is in contrast to a high molecular weight of GM-CSFs derived from lung (Nicola *et al*, 1979), L929 cell (Waheed and Shadduck, 1979) or urine (Slanley and Metcalf, 1971) or *S. japonicum*-infected mouse serum (Owhashi and Nawa, 1985). On the other hand, GM-CSF activity was almost entirely absorbed by Con A-Sepharose 4B and eluted by methyl-α-D-glucoside, suggesting a glycoprotein nature. This feature is held in common with serum-derived GM-CSF from *S. japonicum*-infected mice (Owhashi and Nawa, 1985).

GM-CSF is a potent hematopoietic growth factor which exerts the growth of granulocyte-macrophage linkage. In malaria, G-CSF has enhancing effect of the functional activity of effector cells for protective immunity in malaria (Waki *et al*, 1993). In the present study, elevation of CSF levels in serum was observed during an acute phase of *P. berghei*-infection. GM-CSF appeared in the serum of may contribute the elimination of malaria parasites from the circulation, if not complete, in early-phase malaria.

As to the mechanisms of production of GM-CSF, at least two main possibilities would be generally considered. One is the increased demand of hemopoiesis in malaria. Concerning this, we previously showed that granulocyte-macrophage hemopoiesis is remarkably increased in murine malaria (Asami *et al*, 1992). In the present study, we detected the peak of GM-CSF level at day 2 post-infection, whereas remarkable increase in number of splenocytes began at day 4 post-infection (Asami *et al*, 1992). These facts suggest that the acute increase of CSF level would contributed the rapid increase in number of splenocytes in *P. berghei*-infection. Signs of the increased demand of hemopoiesis may arise by day 2 post-infection. The other possibility is the direct or indirect stimulation of CSF sources, such as macrophages and/or fibroblasts. Concerning this, it has been shown that both IL-1 and TNF can stimulate GM-CSF production by certain types of endothelial and fibroblast cells (Gasson *et al*, 1990). Furthermore, synergistic effect of IL-1 and TNF on GM-CSF production is also shown (Alvaro-Gracia *et al*, 1991). On the other hand, Bate *et al* (1988) showed that malaria parasites could directly stimulate macro-phages for TNF production. Combinations of these findings suggest that malaria parasite may induce CSF production via TNF and/or IL-1 production from macrophages without participation of lymphocytes in the early phase of malaria. The actual role of the serum-derived GM-CSF or the mechanisms of the GM-CSF production should be clarified in future studies.

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