GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR PRODUCTION BY T LYMPHOCYTES IN *PLASMODIUM BERGHEI*-INFECTED MICE

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Abstract. The production of granulocyte-macrophage colony-stimulating factor (GM-CSF) by lymphocytes was examined in murine malaria. When spleen cells or lymph node cells from *P. berghei*-infected mice were cultured *in vitro* with malaria antigen, the GM-CSF production correlated with the incubation time up to 72 hours. When lymphocytes obtained at various days after infection were cultured with the antigen, GM-CSF became detectable as early as 2 days after infection, reached a peak at day 9 and then rapidly decreased. Production of GM-CSF was antigen-specific, and related to the dose of antigen. Treatment of lymphocytes with anti-Thy-1.2 antibody and complement resulted in almost complete loss of GM-CSF-producing activity, while treatment with either anti-CD4 or anti-CD8 antibody and complement resulted in partial loss of GM-CSF-producing activity, indicating that both CD4+ and CD8+ T cells are involved in GM-CSF production in malaria. GM-CSF exhibits glycoprotein nature, and has an apparent molecular weight of 36,000. The molecular properties of this T-cell derived GM-CSF were compared with those of known lymphokine GM-CSF.

INTRODUCTION

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 to the hemopoiesis, recent studies suggest that GM-CSF stimulates neutrophil or macrophage phagocyte system (Moore *et al,* 1981; Fleischmann *et al,* 1986) or the accessory activity of antigen presenting cells (Bowers *et al,* 1990; Owhashi *et al,* 1987). GM-CSF also can stimulate macrophages to produce IL-1 (Moore *et al,* 1980a) or IFN (Moore *et al,* 1989b), cytokines involved in the regulation of inflammatory reaction and/or immune response. In malaria, GM-CSF is involved in the pathogenesis of cerebral malaria (Grau *et al,* 1988). Concerning the change of hemopoiesis in malaria, we (Asami *et al,* 1992) and others (Mungyer *et al,* 1983) showed a remarkable increase in number of hemopoietic stem cells in bone marrow or in spleen. Though these findings suggest the increased production of CSFs, the actual GM-CSF production by T lymphocytes is not fully studied in malaria. In the present study therefore, we examined the production of lymphokine GM-CSF in a lethal murine malaria by using *in vitro* colony assay.

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MATERIALS AND METHODS

Mice and infection

Female C57BL/6 mice, 10-12 weeks old and weighing approximately 20 g at the time of infection, were used throughout this series of experiments. They were infected with *P. berghei* (NK65) by intraperitoneal injection with 1 x 10^6 parasitized erythrocytes. They were killed by cervical dislocation under ether anesthesia.

Parasite antigens

Soluble *P. berghei* antigen was prepared by the method of Singh and Dutta (1992). Soluble extract of *Schistosoma japonicum* eggs and *Dirofilaria immitis* adult worms were prepared by the method as described previously (Horii *et al,* 1988). Protein

concentration was determined by the method of Lowry *et al* (1951).

Preparation of conditioned medium

The spleen or mesenteric lymph node was removed from groups of five mice 8 days after infection, except for the kinetic study. They were gently squashed between two frost-ended slides in cold Hanks' balanced salt solution (HBSS). The cell suspensions were washed with HBSS and suspended in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 2% heat-inactivated fetal bovine serum (FBS, Gibco BRL) and 5 x 10^5 M 2-mercaptoethanol. For removal of adherent cells, the cell suspensions were plated in plastic dishes (Falcon 3001; Becton Dickinson Labware) and incubated at 37°C for 1 hour in a 5% CO_2-air environment. Nonadherent cells were harvested, washed, and suspended in fresh medium at a cell concentration of 5x10^6 /ml, except as otherwise stated. Culturing was carried out at 37°C for 24 hours in a 5% CO_2-air environment. Conditioned medium was obtained by centrifugation at 1,200 g for 10 minutes. The supernatant was sterilized by filtration through a 0.45 μm membrane filter (Millipore Co, Bedford, MA, USA), and stored at -30°C until used.

Cell separations

Plastic-nonadherent lymphocytes from mesenteric lymph nodes were washed twice with HBSS, and suspended in RPMI 1640 containing 0.3% bovine serum albumin and 25 mM N-2- hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES). They were incubated with anti-Thy-1.2 (Serotec), anti-CD4 (Cederlane, Hornby, Ontario, Canada) or anti-CD8 (Cederlane) mAb at 1/500 dilution at room temperature for 30 minutes. After washing twice with HBSS, the cells were further treated with rabbit complement (Low Tox M, Cederlane) at 1/8 dilution with RPMI 1640 containing 0.8% glucose at 37°C for 40 minutes. The cells were washed twice with HBSS and reconstituted in RPMI 1640 supplemented with 3% heat-inactivated FBS.

***In vitro* colony assay**

Details of techniques for colony formation in soft agar have been described previously (Owhashi and Nawa, 1985). Briefly, 1x10^5 bone marrow cells of normal C57BL/6 mice were plated in 35 mm plastic dishes (Falcon 1008; Becton Dickinson Labware, Oxford, CA, USA) in 1 ml of a mixture containing McCoy's 5A medium (Gibco BRL), 0.3% agar (Noble agar; DIFCO, Detroit, MI, USA), 10% FBS (Gibco BRL), and a test sample (200 μl) which was sterilized by Millipore filtration 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).

Affinity chromatography on concanavalin A (Con A)-Sepharose 4B

A Con A-Sepharose 4B column (Pharmacia Fine Chemicals, Uppsala, Sweden) 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, 1mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM Mg Cl_2) containing 0.02% Tween 20. 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 0.2 M methyl-α-D-glucoside.

Size exclusion high performance liquid chromatography (HPLC)

Biologically active fractions from Con A-Sepharose column were concentrated to 0.5 ml and applied to a size exclusion HPLC column (G3000-SW, Toso, Tokyo, Japan) equilibrated with PBS containing 0.02% Tween 20. Chromatography was performed at a flow rate of 1.0 ml /minutes, and fractions of 0.7 ml were collected. The HPLC column was calibrated with the following proteins: ferritin (Boehringer Mannheim, Germany); BSA (Sigma Chemical Co, St Louis, MO, USA); ovalbumin (Sigma); cytochrome c (Sigma); NaCl. The molecular weight of the sample was estimated from a plot of elution time *vs* log (molecular weight).

Heating or pronase treatment

Heating was carried out at 56°C, 80°C or 100°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

Production of GM-CSF from spleen cells or

lymph node cells was examined in *P. bergei*-infected mice at day 8 post-infection. When 5 x 10^6/ml splenocytes or lymph node cells were incubated with 5 μg/ml of *P.berghei* antigen at 37°C, GM-CSF activity in the culture supernatant was proportional to the incubation time up to 72 hours (Fig 1A). Lymph node cells produced higher levels of GM-CSF that spleen cells. When various numbers of spleen cells or lymph node cells were incubated with 5 μg/ml of *P. berghei* antigen at 37°C for 24 hours, the peak of GM-CSF production was observed at around 5 x 10^6 cells per ml (Fig 1B).

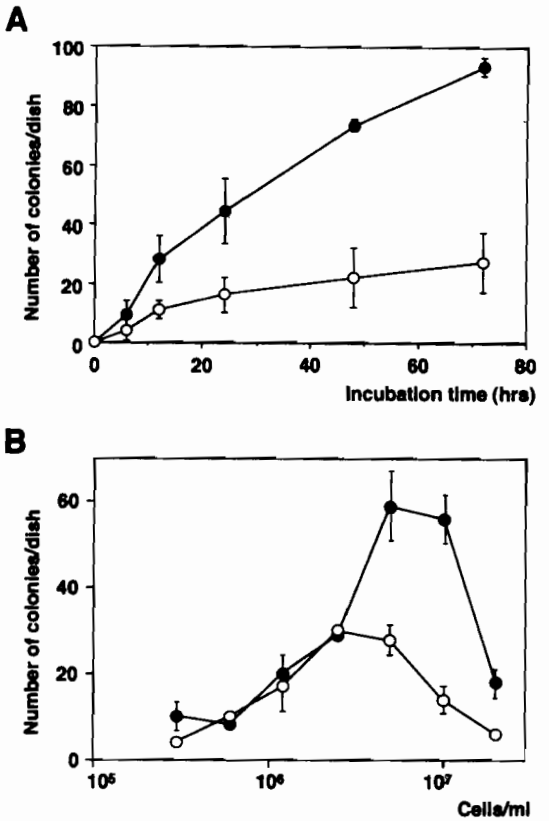

Fig 1–Production of GM-CSF from splenocytes or lymph node cells in *P. berghei*-infected mice at day 8 post-infection. A. Time course of GM-CSF production. Splenocytes (O) or lymph node cells (●) were incubated with 5 μg/ml of *P. berghei* antigen at the cell concentration of 5 x 10^6/ml at 37°C. B. Production of GM-CSF at different cell concentrations. Various concentrations of splenocytes (O) or lymph node cells (●) were incubated with 5 μg/ml of *P. berghei* antigen at 37°C for 24 hours. GM-CSF activity was expressed as the number of colonies generated per dish (mean ± SD).

Drastic decrease of GM-CSF production was observed at 2 x 10^7 cells per ml.

To study the kinetic change of GM-CSF production, spleen cells or lymph node cells obtained from individual mouse at various times after infection were cultured with *P. berghei* antigen. The conditioned media were harvested 24 hours later, and their CSF activity was measured. As shown in Fig 2, GM-CSF activity became detectable as early as day 2 post-infection. The peak of GM-CSF production was observed at day 9 post-infection. GM-CSF production became undetectable at day 14 post-infection.

To examine the antigen specificity of lymphocytes for GM-CSF production, lymph node cells day 8 post-infection were incubated with various concentrations of antigens of *P. berghei* or other parasites. As shown in Fig 3, increase in GM-CSF production was observed when lymph node cells were incubated with *P. berghei* antigen. In contrast, incubation with *D. immitis* or *S. japonicum* antigen had no effect on the production of GM-CSF. *P. berghei* antigen itself had no GM-CSF activity.

To examine the surface phenotype of GM-CSF producing cells, lymph node cells or spleen cells obtained from *P. berghei*-infected mice were

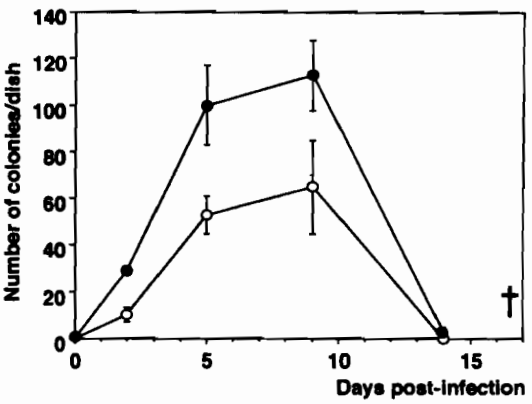

Fig 2–Kinetic change of GM-CSF production of splenocytes (O) or lymph node cells (●) after *P. berghei*-infection. Splenocytes or lymph node cells obtained from individual mice at various times after infection were cultured with 5 μg/ml of *P. berghei* antigen at the cell concentration of 5 x 10^6/ml at 37°C. GM-CSF activity was expressed as the number of colonies generated per dish (mean ± SD).

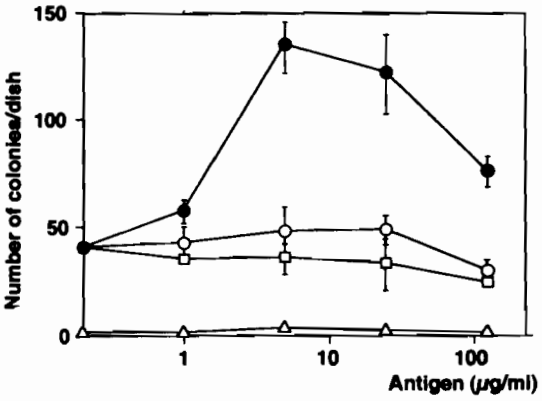

Fig 3–Antigen specificity of GM-CSF production of lymph node cells. Lymph node cells from *P. berghei*-infected mice at day 8 post-infection were incubated with soluble antigen of *P. berghei* (●) *S.japonicum* (O) or *D. immitis* (❑). GM-CSF activity of *P. berghei*-antigen alone (Δ) is also shown. GM-CSF activity was expressed as the number of colonies generated per dish (mean ± SD).

treated with anti-Thy-1.2, anti-CD4 or anti-CD8 mAb, and complement. After the treatments, GM-CSF production was examined in the presence of *P. berghei* antigen. As shown in Fig 4, most of the GM-CSF production was abolished by anti-Thy-1.2 treatment, whereas approximately half of GM-CSF production retained by either anti-CD4 or anti-CD8 mAb treatment. These results suggest that both $CD4^+$ T cells and $CD8^+$ T cells are involved in the GM-CSF production in malaria.

To study the physicochemical properties of GM-CSF, 2.0 ml of 50 times concentrated spleen cell conditioned medium was applied on Con A-Sepharose 4B, and eluted with a competitive sugar : methyl-α-D-glucoside (Fig 5). Approximately 60% of GM-CSF was adsorbed onto Con A-Sepharose 4B and was eluted by methyl-α-D-glucoside, indicating its glycoprotein nature. The bound materials were concentrated, and separated by HPLC equipped with G3000SW column. As

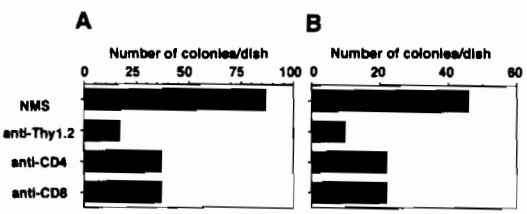

Fig 4–Surface phenotype of the GM-CSF producing cells of *P. berghei*-infected mice. Lymph node cells (Panel A) or spleen cells (Panel B) obtained from *P. berghei*-infected mice at day 6 after infection were treated with 500 times diluted normal mouse serum (NMS), anti-Thy-1.2, anti-CD4 or anti-CD8 mAb, followed by with complement. After treatment, the cells were washed and reconstituted to make up a final cell concentration of 5×10^6/ml RPMI 1640 medium containing 5 mg/ml of *P. berghei* antigen. After incubation at 37°C for 24 hours, GM-CSF activity was measured.

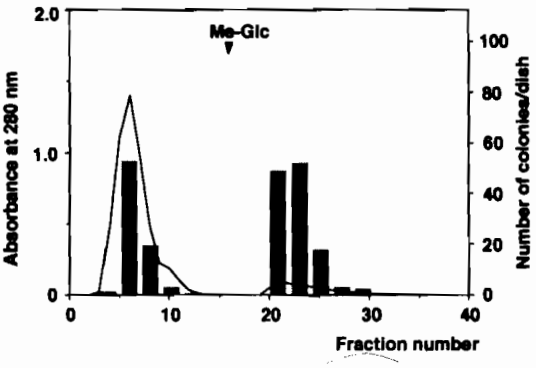

Fig 5–Con A-Sepharose 4B affinity chromatography of GM-CSF. Conditioned medium was prepared with spleen cells of *P. berghei*-infected mouse at day 8 after infection. Fifty times concentrated condition medium (2 ml) was applied on Con A-Sepharose 4B column. Bound material was eluted by Con A buffer containing 0.2 M methyl α-D-glucoside (Me-Glc). Absorbance at 280 nm (–) or GM-CSF activity (▭) of each fraction is shown. The activity of GM-CSF is expressed as the mean number of colonies generated per dish. Bound material was eluted with Con A buffer containing 0.2 M methyl α-D-glucoside (Me-Glc).

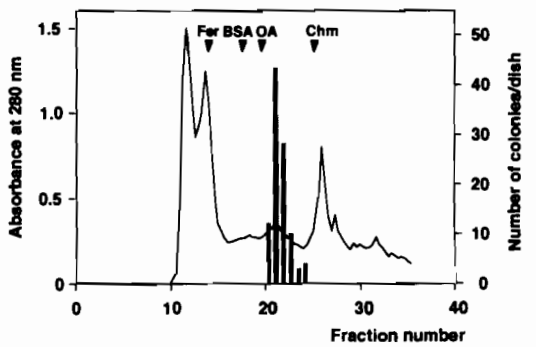

Fig 6–High performance liquid chromatography of GM-CSF. Con A-Sepharose adsorbed GM-CSF was concentrated, and analyzed by HPLC equipped with G3000SW column. Absorbance at 280 nm (–) or GM-CSF activity (▭) of each fraction is shown. GM-CSF activing is expressed as the mean number of colonies generated per dish.

shown in Fig 6, GM-CSF activity was detected as a single peak with an apparent molecular weight of 36,000. GM-CSF was stuble to heating at 56°C and relatively stable to heating at 80°C, but was sensitive to heating at 100°C or pronase digestion (Table 1).

Table 1

Effect of heating or treatment with pronase on GM-CSF activity.

Treatments		No. of colonies/dish*
A		
Control		88 ± 3
Heating	56°C	82 ± 2
	80°C	59 ± 2
	100°C	0 ± 1
B		
Control		87 ± 2
Pronase		2 ± 1

*GM-CSF activity was expressed as the number of colonies generated per dish (mean ± SD)

DISCUSSION

Splenomegaly is one of the characteristic features of malaria in human and experimental animals. We previously showed the rapid increase of hematopoietic stem cells in spleen in acute malaria (Asami *et al,* 1992, 1993). In the present study, we showed the production of GM-CSF from T lymphocytes in spleen as well as lymph nodes in murine malaria. Kinetic study showed the GM-CSF production by T lymphoxytes peaked at day 9 post-infection (Fig 2). This coincides with the peak of total number of spleen cells in *P. berghei*-infection (Asami *et al,* 1992). Taking these data together, a remarkable increase of granulocyte-macrophage hemopoiesis occurs in the spleen during acute malaria, which could partially account for the mechanisms of splenomegaly.

As sources of GM-CSF, monocytes-macrophages (Eaves and Bruce, 1974) or fibroblasts (Waheed and Shadhuck, 1979) are well-known. In addition, T lymphocytes are also shown to produce GM-CSF upon stimulation with various mitogens (Parker and Metcalf, 1974). Furthermore, GM-CSF production by a T-cell hybridoma (Burgess *et al,* 1981), thymoma cell line (Hilflker *et al,* 1981) or T-cell clones (Prystowsky *et al,* 1983; Bruserud *et al,* 1993) after stimulation with Con A or specific antigens have been reported. In malaria, Lucas *et al,* (1995) showed an increased level of mRNA expression of GM-CSF in acute *P. yoelii*-infection. Singh and Dutta (1992) showed that normal macrophages could produce GM-CSF upon stimulation with a soluble malaria antigen. In the present study, we showed that T lymphocytes could produce the biologically active GM-CSF in antigen specific manner in *P.berghei*-infection. Negative selection study revealed that both $CD4^+$ and $CD8^+$ T cells were involved in GM-CSF production in malaria. In fact, both $CD8^+$ and $CD4^+$ clones are shown to produce GM-CSF (Bruserud *et al,* 1993).

In the present study, larger part of GM-CSF bound to Con A-Sepharose, indicating its glycoprotein nature. GM-CSF detected as a single peak in size exclusion high performance liquid chromatography with an apparent molecular weight of 36,000 (Fig 6). GM-CSF that did not bind to Con A-Sepharose had a similar molecular weight (data not shown). The molucular weight of the GM-CSF coincided with T lymphocyte-derived CSF (MW 34,000) reported by others (Lusis *et al,* 1981), but was obviously larger than the calculated one from the putative amino acid sequence from cDNA (Miyatake *et al,* 1985), due to the presence of up to 52% carbohydrate by weight in the molecule. In contrast to the T cell derived GM-CSF, higher molecular weight GM-CSFs are shown in lung (Nicola *et al,* 1979), L929 cell (Waheed and Shadduck, 1979), urine (Slanley and Metcalf, 1971) or *S. japonicum*-infected mouse serum (Owhashi and Nawa, 1985). The glycoprotein nature of GM-CSF is, however, held in common with these GM-CSFs.

GM-CSF is a potent hematopoietic growth factor which exerts the growth of granulocyte-macrophage linkage. In addition, GM-CSF is shown to have a broad range of regulatory fuctions on macrophages and neutrophils. For example, GM-CSF enhances the accessory activity of antigen presenting cells (Bowers *et al,* 1990, Owhashi *et al,* 1987), production of cytokines such as IL-1 (Moore *et al,* 1980a) or IFN (Moore *et al,* 1989b). Indeed, Lelchuk *et al* (1984) showed the increased level of IL-1 production from the adherent cells of spleen of

P. yoelii-infected mice upon stimulation with LPS. Furthermore, GM-CSF or G-CSF could enhance neutrophil (Moore *et al,* 1981; Fleischmann *et al,* 1986, Lopez *et al,* 1983) or macrophage (Grabstein *et al,* 1986)-mediated cytotoxity. Related to this Waki *et al,* (1993) and waki (1994) showed that G-CSF could augment neutrophil mediated cytotoxicity on malaria parasite. In addition, GM-CSF could augment the proliferative response of T lymphocytes or induction of lymphocyte-activated killer (LAK) cells by IL-2 (Stewart-Aker *et al,* 1993). As for the localization of hemopoietic stem cells, GM-CSF or G-CSF is shown to contribute the mobilization of stem cells in peripheral blood. Related to this, we previously showed a remarkable increase in number of hemopoietic stem cells in spleen as well as in bone marrow during acute phase of murine malaria (A- sami *et al,* 1992a, b). The increased stem cells in spleen may be derived from bone marrow via circulation by the effect of the increased GM-CSF. Taken these findings together, GM-CSF production from T cells may contribute to the protective immunity or immunophathogenesis in this disease. The actual role of the T cell derived-GM-CSF in malaria should be clarified in future.

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