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Granulocyte–macrophage colony-stimulating activity in the serum of mice stimulated with homogenates of *Trypanosoma gambiense*

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SUMMARY

Infection of mice with *Trypanosoma gambiense* induced a rapid and transient increase in levels of serum granulocyte–macrophage colony-stimulating activity (GM–CSA) followed by an increase in the numbers of circulating neutrophils and large mononuclear cells. Similar phenomena were observed in mice injected with a trypanosomal homogenate. In such mice, levels of GM–CSA were markedly elevated as early as 1–2 days after administration of the homogenate and then rapidly decreased. Numbers of circulating neutrophils and large mononuclear cells increased within 2 and 3 days after injection of the homogenate, respectively. Induction of GM–CSA by the trypanosomal homogenate promoted colony formation by normal spleen cells, but did not support colony formation by spleen cells from mice treated with 5-fluorouracil. Serum levels of GM–CSA were elevated by injection of crude trypanosomal membrane fractions, but not by soluble components found in supernatants from centrifuged trypanosomal homogenates. GM–CSA-inducing ability of the crude membrane fraction was sensitive to heating at 60°C for 30 min, treatment at pH 2·0, pronase digestion, periodate oxidation, and exposure to 0·1% sodium dodecyl sulphate. This biological activity was also reduced by trypsinization of living trypanosomes before preparation of membrane fractions. These findings suggest that the active component of parasites to induce GM–CSA is associated with a glycoprotein in the surface coat of *T. gambiense*. GM–CSA may be related to the haematological and immunopathological alterations that occur in African trypanosomiasis.

Keywords *Trypanosoma gambiense* African trypanosomiasis granulocyte–macrophage colony-stimulating activity

INTRODUCTION

The outstanding immunopathological effects of African trypanosome infection are profound proliferation of T and B cells, macrophages, and null cells in the spleen and bone marrow (Murray *et al.*, 1974; Mansfield & Bagasra, 1978; Mayor-Withey *et al.*, 1978; Morrison *et al.*, 1978). In addition, leucocytosis including neutrophilia, plasmacytosis or monocytosis has been reported in humans and in experimental animals (Clayton *et al.*, 1980; Wéry *et al.*, 1982; Poltera, 1985). Certain host-cell derived soluble mediators may be involved in these alterations of the immune system (Bancroft *et al.*, 1983; Fierer, Salmon & Askonas, 1984; De Gee, Sonnenfeld & Mansfield, 1985; Mitchell, Pearson & Gauldie, 1986). It has been reported that whole or fractionated trypanosomal homogenates can mimic some immunological alterations observed in infected mice (Hudson *et al.*, 1976; Clayton *et al.*, 1979; Sacks *et al.*, 1982; Diffley, 1983; Oka, 1986). Recently, we found that host-cell derived soluble mediators related to the induction of polyclonal B cell activation are released into the serum of mice injected with homogenates of *Trypanosoma gambiense* (Oka & Ito, 1987).

In the present study, the induction of granulocyte–macrophage colony-stimulating activity (GM–CSA) was examined in mice injected with whole and fractionated homogenates of *T. gambiense*. Colony-stimulating factors have been shown to exhibit various effects on the function of macrophages and neutrophils (Handman & Burgess, 1979; Kurland *et al.*, 1979; Lin & Gordon, 1979; Moore *et al.*, 1980a, 1981; Weisbart *et al.*, 1985; Grabstein *et al.*, 1986) as well as exerting a major regulatory effect on granulocyte and macrophage-monocyte formation (Bradley & Metcalf, 1966; Metcalf, 1985). Furthermore, the induction of GM–CSA in serum has been reported in bacterial (Trudgett, McNeil & Killen, 1973), viral (Foster, Metcalf & Kirchmyer, 1968) and parasitic (Owashi & Nawa, 1985) infections. Our findings suggest an association of a substance (or substances) responsible for the induction of GM–CSA with a glycoprotein moiety that is present in the surface coat of *T. gambiense*.

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MATERIALS AND METHODS

Mice

Female ddY mice, bred under specific pathogen-free conditions at Tokushima Experimental Animal Laboratory (Tokushima, Japan), were used at ages of 7 to 10 weeks.

Trypanosome preparation

T. gambiense (Wellcome strain) was passaged in mice every 3 days. Trypanosomes were separated from whole heparinized blood on a DEAE-Sephadex A-25 column equilibrated with phosphate-buffered saline (PBS) glucose (Lanham, 1968). Purified parasites were washed three times with PBS and disrupted by repeated freeze-thawing, as described previously (Oka *et al.*, 1984). This homogenate was centrifuged at 12 000 *g* for 30 min to remove nuclei, kinetoplasts and large debris (Sacks *et al.*, 1982). The supernatant was then centrifuged at 144 000 *g* for 1 h to prepare the final supernatant and crude membrane fractions.

Trypsin treatment of trypanosomes

An aliquot of viable trypanosomes (10^9 organisms/ml) was incubated with 50 μg/ml trypsin (type IV; Sigma Chemical Co., St Louis, MO) in PBS for 10 min at 37°C. The reaction was terminated by the addition of 1 mM tosyl-L-lysin chloromethylketone. The trypsin-treated parasites were washed by centrifugation three times with PBS at 1000 *g* for 10 min. A crude membrane fraction of trypsin-treated parasites was prepared as described above. Even after tryptic digestion, trypanosomes were not ruptured and their motility was as active as that of untreated trypanosomes. Electron micrographs of trypsin-treated parasites showed that the surface coat which forms a closely packed layer on the plasma membrane was mostly removed without causing any other morphological changes (Oka, 1986). Protein concentration was measured by the method of Lowry *et al.* (1951).

Treatment of crude membrane fraction

The crude trypanosome membrane fraction was dialysed against either sodium acetate buffer (pH 2·0) or PBS (pH 7·4) for 12 h and then against PBS for 12 h. Heating of the sample at 60°C was carried out in a water-bath for 30 min. Other samples were treated with 0·1% sodium dodecyl sulphate for 12 h followed by dialysis against PBS for 12 h. Periodate treatment was carried out in the dark with 25 mM sodium metaperiodate at 4°C for 24 h followed by dialysis against PBS for 12 h. For pronase treatment, the membrane fraction was incubated with 100 μg/ml pronase at 37°C for 6 h just before injection into mice.

Preparation of serum

Mice were infected intraperitoneally with 10^3 washed, viable parasites in 0·2 ml of PBS. Since the Wellcome trypanosome strain kills mice within 4–5 days at this dose, mice were cured with the trypanocidal drug Ganaseg (4,4′-diazoaminodibenzamidine diaceturate, 150 μg/ml, E.R. Squibb and Sons, Prinstone, NJ) 3 days after infection. Trypanosomes were not detectable in the blood within 1 day after the treatment. In another experiment, mice were injected intraperitoneally with various doses of the whole trypanosomal homogenate or with various doses of the subcellular fractions. Serum was collected at various time intervals after infection or injection with the homogenates or subcellular fractions, and the pooled sera (5 mice per pool for each dose level) were sterilized by filtration through a 0·45-μm micropore filter and stored at −70°C until use.

Peripheral blood leucocyte counts

At various intervals after infection or administration of the trypanosomal homogenate or serum, the numbers of leucocytes in the peripheral blood were measured from tail vein samples. Smears of tail blood were stained with Giemsa for differential leucocyte counting.

In vitro *colony assay*

Bone marrow cell culture was performed by the soft agar method (Bradley & Metcalf, 1966) with a slight modification. Briefly, 10^5 cells were plated in 35-mm plastic dishes (Falcon 3001, Becton Dickinson Labware, Lincoln Park, NJ) in 1 ml of 0·3% agarose in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY) and 200 μl of two-fold-diluted test sample. Cultures were incubated for 7 days at 37°C in a humidified atmosphere of 7% CO_2 in air. For assay of colony formation of pluripotent stem cells, mice were injected intravenously with 5-fluorouracil (5-FU; 150 mg/kg). Spleen cells were harvested 4 days later and 10^6 cells were cultured as described above with the exception that 5×10^{-5} M 2-mercaptoethanol were added to the medium. Colonies (aggregates of 50 or more cells) were scored using a dissection microscope. A cytochemical staining method was employed for differential counting of colonies (Hayama, Nawa & Kotani, 1985). The agar gels were dried on glass slides and stained by the β-glucuronidase-positive macrophage–monocyte colonies, enzyme-negative granulocyte colonies, or mixed types. All assays were done with quadruplicate cultures and repeated at least twice.

Statistical analysis

The statistical significance of the data were determined by Student's *t*-test. *P* values of less than 0·05 were considered significant.

RESULTS

Changes in serum GM–CSA levels and peripheral leucocyte counts after infection of mice with T. gambiense

Changes in levels of serum GM–CSA were examined in relation to changes in the total leucocyte and differential cell counts after trypanosome infection (10^3 parasites). Levels of serum GM–CSA transiently increased on day 2 post-infection, when parasites became detectable in the blood and then rapidly decreased to the levels of uninfected controls. One day after the treatment with trypanocidal drug, levels of serum GM–CSA again increased transiently (Fig. 1a). Cytochemical analysis of the colonies revealed that those generated by addition of day-2 serum were composed of macrophages (63%), granulocytes (25%) or mixed types (12%). As shown in Fig. 1b, the total leucocyte count progressively decreased on day 2 post infection and then gradually increased. Analysis of the differential cell types showed that the number of neutrophils significantly increased 1 day post infection and remained at higher levels throughout the experiment. Numbers of large mononuclear

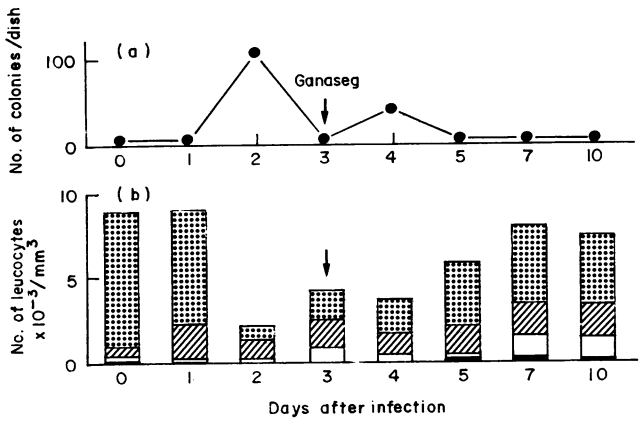

Fig. 1. Changes in serum granulocyte-macrophage colony-stimulating activity levels (a) and peripheral blood leucocyte counts (b) in mice infected with 10^3 *T. gambiense*. Mice were cured of infection by Ganaseg treatment (150 μg/mouse) on day 3 of infection (arrow). Each point represents the mean ± s.e.m. for quadruplicate assays. Standard error is concealed behind each point. Each column represents the mean for five mice. ▩, lymphocytes; ■, neutrophils; □, large mononuclear cells; and ■, eosinophils. Statistical significance ($P < 0.05$) exists between control mice (day 0) and infected mice on the following days: (lymphocytes) 2 to 10; (neutrophils) 1 to 2, and 4 to 10; and (large mononuclear cells) 3 and 7 to 10.

cells were also significantly higher on day 3 and on days 7–10 post infection. A marked lymphopenia appeared on day 2 post infection. During the remainder of the experiment, the number of lymphocytes gradually rose to levels slightly less than those at day 0. Numbers of eosinophils were unchanged throughout the experiment.

Changes in serum GM-CSA levels and peripheral leucocyte counts after injection of mice with homogenates of T. gambiense

After the administration of trypanosomal homogenates, serum levels of GM-CSA increased as early as 1 day after administration, reached a peak at day 2, and then progressively decreased (Fig. 2a). The colonies generated by day-2 serum were macrophages (58%), granulocytes (15%) or a mixture of both cell types (27%), as shown by cytochemical analysis. There was a possibility that elevated levels of GM-CSA were due simply to the parasite-derived substances. However, addition of various doses of the trypanosomal homogenate to an *in vitro* colony assay system did not induce colony formation (data not shown). Furthermore, when the serum was tested with polyspecific antibodies to the trypanosomal homogenate by immunoelectroblotting analysis, parasite-derived protein bands were not detectable in the preparation (Oka & Ito, 1987). As shown in Fig. 2b, leucocyte numbers transiently decreased 2 days after administration of the homogenate and then increased gradually. Morphological analysis showed that the numbers of neutrophils and large mononuclear cells markedly increased on days 2 and 3, respectively. Transient lymphopenia was observed on days 2–3 and then lymphocyte numbers began to increase until they exceeded those of untreated controls. Numbers of eosinophils were unchanged during the course of the experiment.

Changes in peripheral leucocyte counts after transfer of serum from mice injected with trypanosomal homogenates

We further examined whether the haematological changes described above were also induced by transfer of serum from mice injected with trypanosomal homogenate into normal

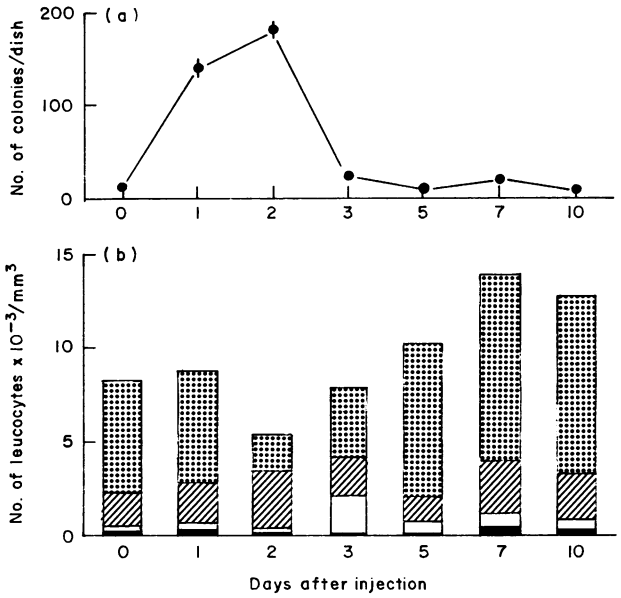

Fig. 2. Changes in serum granulocyte-macrophage colony-stimulating activity levels (a) and peripheral blood leucocyte counts (b) in mice injected with 400 μg protein of *T. gambiense* cell homogenate. Each point represents the mean ± s.e.m. for quadruplicate assays. Each column represents the mean for five mice. ▩, lymphocytes; ■, neutrophils; □, large mononuclear cells; and ■, eosinophils. Statistical significance ($P < 0.05$) exists between control mice (day 0) and mice injected with trypanosomal homogenates on the following days: (lymphocytes) 2, 5 and 10; (neutrophils) 2 and 10; and (large mononuclear cells) 3 and 7.

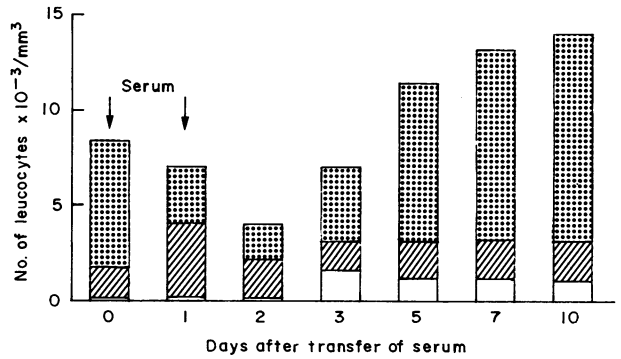

Fig. 3. Changes in peripheral blood leucocyte counts in mice injected with sera. Sera were obtained from mice 2 days after administration of *T. gambiense* cell homogenate (100 μg protein), and 0·1 ml of sera was transferred into normal mice (arrow). Each column represents the mean for five mice. ▩, lymphocytes; ■, neutrophils; □, large mononuclear cells. Statistical significance ($P < 0.05$) exists between control mice (day 0) and mice injected with sera on the following days: (lymphocytes) 1 to 3 and 7 to 10; (neutrophils) 1 to 2; and (large mononuclear cells) 3 to 10.

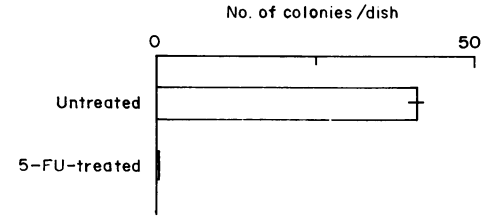

Fig. 4. Colony formation of spleen cells from mice treated with 5-fluorouracil (5-FU). Sera for colony assay were obtained from mice 2 days after injection of *T. gambiense* cell homogenate (100 μg protein). Values represent the mean ± s.e.m. for quadruplicate assays.

recipient mice. Two days after injection of the homogenate, serum was collected and transferred into recipients. As we observed in mice infected with trypanosomes or injected with trypanosomal homogenates, leucocyte numbers markedly decreased in recipients 2 days after serum transfer and then increased profoundly. The number of neutrophils significantly increased 1 day after serum transfer, and the number of large mononuclear cells increased 3–10 days after transfer. Lymphopenia was observed 1–3 days after transfer and lymphocyte numbers progressively increased thereafter (Fig. 3). Significant changes in the total leucocyte numbers and differential cell counts were not observed when mice were injected with normal serum (data not shown). These results may suggest that the haematological alterations are mediated by serum factors, and that serum levels of GM-CSA are closely related to these alterations.

Effect of serum GM-CSA on colony formation of pluripotent stem cells

In order to determine the target cells of the serum GM-CSA, spleen cells from mice treated with 5-FU were used as target cells. As shown in Fig. 4, serum obtained from mice 2 days after injection of the trypanosomal homogenate supported formation of colonies of normal spleen cells, but did not generate colonies from 5-FU-treated mouse spleen cells, indicating that serum levels of GM-CSA could not stimulate 5-FU-resistant pluripotent stem cells.

GM-CSA-inducing ability of subcellular trypanosome fractions

Subcellular components were prepared by centrifugation of freeze-thawed trypanosomes in order to characterize an active

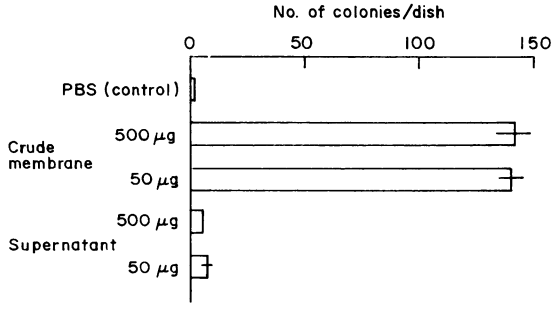

Fig. 5. Ability of trypanosome subcellular fractions to induce granulocyte-macrophage colony-stimulating activity. Sera for colony assay were obtained 2 days after injection of the subcellular fractions. Values represent the mean ± s.e.m. for quadruplicate assays. PBS, phosphate-buffered saline.

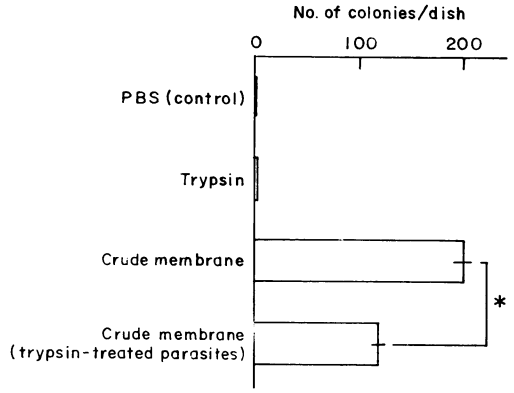

Fig. 6. Ability of crude membrane fraction prepared from trypsin-treated trypanosomes to induce granulocyte-macrophage colony-stimulating activity. Sera for colony assay were obtained from mice 2 days after injection of the crude membrane fraction (50 μg protein) prepared either from untreated or trypsin-treated trypanosomes. Values represent the mean ± s.e.m. for quadruplicate assays. *, $P < 0.005$. PBS, phosphate-buffered saline.

component (or components) responsible for induction of GM-CSA. The active component was retained exclusively in a crude membrane faction sedimenting between 12 000 and 144 000 ***g***. In contrast, the 144 000 ***g*** supernatant fraction was devoid of activity (Fig. 5). In addition, the ability of the crude membrane fraction to induce GM-CSA was significantly reduced by trypsinization of viable trypanosomes prior to the preparation of membrane fraction (Fig. 6).

Effects of physicochemical treatments on the ability of trypanosomes to induce GM-CSA

Some of the physicochemical properties of the trypanosome GM-CSA-inducing substance(s) were examined. As shown in

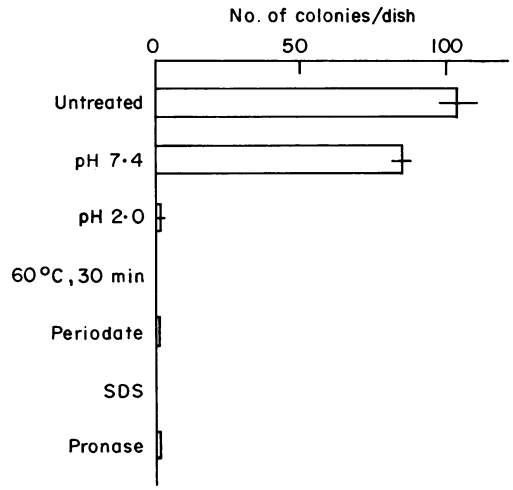

Fig. 7. Effects of various treatments on the ability of the crude membrane fraction of *T. gambiense* to induce granulocyte-macrophage colony-stimulating activity. Treatments of the crude membrane fraction were as described in Materials and Methods. Serum for colony assay was obtained 2 days after injection of the crude membrane fraction (100 μg protein). Values represent the mean ± s.e.m. for quadruplicate assays. SDS, sodium dodecyl sulphate.

Fig. 7, the ability of the crude membrane fraction to induce GM–CSA was completely abrogated by treatment at pH 2·0, heating at 60°C for 30 min, periodate oxidation, pronase digestion or solubilization with 0·1% sodium dodecyl sulphate, indicating its membrane-binding glycoprotein nature.

DISCUSSION

African trypanosomes cause remarkable pathological and immunological alterations in the infected host. The mechanism by which parasite infection interferes with the host immune system is still not clearly understood. Infection with trypanosomes results in changes in the levels of soluble mediators such as the interleukins (Mitchell *et al.*, 1986), prostaglandins (Fierer *et al.*, 1984) and interferons (Bancroft *et al.*, 1983; De Gee *et al.*, 1985). Immunoregulatory mediators are essential for the cytokine cascade that is involved in the induction of inflammatory and immune responses. Thus, the immunopathological changes that accompany trypanosome infections will be created through alterations in the production of cytokines.

The results presented here show that infection of mice with *T. gambiense* or injection of trypanosomal homogenates into mice causes a rapid and transient elevation of serum levels of GM–CSA. A similar phenomenon has been also observed by many investigators after viral, bacterial and parasitic infections (Foster *et al.*, 1968; Trudgett *et al.*, 1973; Owashi & Nawa, 1985). Therefore, there is a possibility that GM–CSA detected in serum is either exerted by a residual parasite component (or components) or induced by a viral or endotoxic contaminant of the parasite preparation. However, this possibility is unlikely for the following reasons: (i) the trypanosomal homogenate itself did not induce *in vitro* colony formation; (ii) the ability of homogenates to induce GM–CSA was abolished by heating (60°C for 30 min) or pronase digestion—two treatments that have little or no effect on endotoxin; (iii) GM–CSA was produced by the injection of membrane fractions, but not supernatant fractions in which viruses or endotoxic components may be contained; and (iv) an immunoelectroblotting analysis using polyspecific antibodies to the trypanosomal homogenate was unable to detect parasite-derived proteins in the serum of mice treated with trypanosomal homogenates.

Although we have data on dose-response effects of trypanosomal homogenate on the induction of GM–CSA in serum, we have not presented them in the results. To examine the relation between the dose of trypanosomal homogenate and serum levels of GM–CSA, mice were given various doses of the homogenate (500–5 μg/mouse) and 2 days later the serum was collected to measure the GM–CSA levels. The injection of less than 5 μg protein of the homogenate failed to induce detectable levels of GM–CSA in serum. The generation time of Wellcome strain of trypanosomes is about 7 h (Yabu & Takayanagi, 1986), and 10^8 trypanosomes are equivalent to approximately 775 μg protein of the homogenate. When mice are infected with 10^3 parasites, the number of parasites will increase mathematically up to $1{\cdot}3 \times 10^5$ trypanosomes/mouse by day 2 after infection; $1{\cdot}3 \times 10^5$ trypanosomes are equivalent to about 1 μg protein of homogenate, the dose of which does not induce GM–CSA. From these data, it is conceivable that the active infection is more effective on the induction of GM–CSA than is injection of the homogenate.

The present study indicates that the parasite-derived component that is capable of inducing GM–CSA is membrane bound and glycoprotein in nature. Alternatively, the lack of GM–CSA-inducing activity in supernatant fractions of trypanosomal homogenates may be due to inadequate interaction of the active components with host cells. It has been reported that polyclonal B cell activation is induced by variant surface glycoproteins of trypanosomes (Diffley, 1983). Since trypsinization of living parasites prior to homogenation and subcellular fractionation reduces the ability of parasites to induce GM–CSA, an active component responsible for induction of GM–CSA seems to be present in the dense surface coat which is on the plasma membrane of trypanosomes. Further studies will be necessary to clarify whether induction of serum GM–CSA and polyclonal B cell activation are caused by the same parasite component.

An increase in the number of peripheral granulocytes and large mononuclear cells was associated with the elevation of serum GM–CSA levels. The increase in the serum GM–CSA seems to be the result of direct stimulation of GM–CSA-producing cells by active trypanosome-derived stimulant(s). It has been suggested that only monocyte–macrophages can produce GM–CSA (Moore & Williams, 1972; Eaves & Bruce, 1974). However, it has recently been established that many mouse tissues, non-haemopoietic cell lines and stimulated lymphoid cells produce and can release biologically similar GM–CSA (Burgess & Metcalf, 1980). Potent stimulants of the mononuclear phagocyte system are known to cause elevation of GM–CSA in serum (Metcalf, 1971; Hayama *et al.*, 1985). During the course of trypanosomiasis, the mononuclear phagocyte system is in an activated state in terms of histological criteria and phagocytic activity (Murray *et al.*, 1974; Fierer & Askonas, 1982). In addition, phenotypic changes associated with macrophage activation have also been observed (Grosskinsky *et al.*, 1982). Thus, the cells in the mononuclear phagocyte system are at least in part a possible source of GM–CSA production.

To date, it is recognized that the formation of colonies by granulocyte–macrophage lineage cells is under the influence of four kinds of colony-stimulating factors—multi-(interleukin-3); granulocyte/macrophage; granulocyte; and macrophage. It is still not clear to what extent these colony-stimulating factors contribute to colony formation in our experimental system. The GM–CSA in our system did not support colony formation by spleen cells from mice treated with 5-FU, suggesting that this GM–CSA does not act on 5-FU-resistant pluripotent stem cells. Furthermore, we found that GM–CSA could not support the proliferation of multi-colony-stimulating factor-dependent cell line FDC-P2 (data not shown). These observations indicate that multi-colony-stimulating factor is not involved in colony formation in our experiments.

Neutrophilia occurs after resolution of the initial peak of parasitemia (Clayton *et al.*, 1980). A marked increase in the macrophage content of lymphoid organs and in the numbers of blood monocytes and peritoneal macrophages is also evident (Murray *et al.*, 1974; Clayton *et al.*, 1980). In addition, splenomegaly accompanied by an increase of macrophage numbers is a characteristic feature of trypanosome infection (Mansfield & Bagasra, 1978). These observations and our present results suggest that GM–CSA may, at least in part participate in the haematological and immunopathological phenomena that occur during trypanosomiasis.

In mice infected with *T. brucei*, Clayton *et al.* (1980) observed transient lymphopenia within 2 days after the infec-

tion, and a marked increase in circulating lymphocytes after resolution of the initial peak of parasitemia. However, little is known about the mechanisms that induce these haematological alterations. Our experiments suggest that a host-cell derived factor (or factors) is involved in these phenomena, since transfer of serum as well as injection of trypanosomal homogenates caused transient reduction of lymphocyte numbers followed by a profound expansion in population size. We previously reported that a polyclonal B cell activating factor(s) is induced in the serum of mice injected with the trypanosomal homogenates (Oka & Ito, 1987). This factor may directly or indirectly contribute to lymphocytosis.

In addition to the functional importance as a proliferative or differentiative stimulus for granulocyte and macrophage production, GM-CSA can potentiate various functional activities of these cells (Moore *et al.*, 1981; Weisbart *et al.*, 1985). Stimulated macrophages exhibit increased phagocytic activity, or increase their production of prostaglandins, plasminogen activator, interleukin-1 and interferon (Handman & Burgess, 1979; Kurland *et al.*, 1979; Lin & Gordon, 1979; Moore *et al.*, 1980a, b). As mentioned earlier, elevation of macrophage phagocytic activity and production of both prostaglandins and interferons occur in mice infected with trypanosomes. It is therefore conceivable that GM-CSA could contribute directly and/or indirectly to the production of these immunoregulatory and inflammatory mediators. Thus, GM-CSA would be involved in the mechanisms that cause immunological and immunopathological alteration during African trypanosomiasis. Further studies will be necessary to gain more detailed information about the possible role of GM-CSA in trypanosome infection.

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