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Granulocyte-Macrophage Colony-Stimulating Factor: Involvement in Control of *Trypanosoma cruzi* Infection in Mice

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Several cytokines play crucial roles in *Trypanosoma cruzi* infection in mice, but the involvement of endogenous granulocyte-macrophage colony-stimulating factor (GM-CSF) is poorly documented. This report shows that *T. cruzi* infection of mice triggered an early and sharp increase in plasma GM-CSF during the ascending phase of parasitemia. The plasma GM-CSF concentration remained stable at the peak of parasitemia and subsequently increased in those mice that survived to the acute phase. GM-CSF level increased again sharply, while parasitemia was rapidly decreasing. Finally, GM-CSF was undetectable, soon after the disappearance of circulating parasites. Injection of *T. cruzi*-infected mice with neutralizing anti-GM-CSF monoclonal antibodies induced the early appearance of parasitemia and aggravated cumulative mortality. In contrast, recombinant mouse GM-CSF (rmGM-CSF) caused sharp decreases in both parasitemia and cumulative mortality in *T. cruzi*-infected mice. Peritoneal macrophages from rmGM-CSF-treated and infected or uninfected mice were less infected ex vivo than those from control mice. Taken together, these data demonstrate the protective action of endogenous GM-CSF in *T. cruzi* infection. Neutralization of endogenous GM-CSF aggravates infection, while exogenous rmGM-CSF decreases both parasitemia and host mortality.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine responsible for the proliferation, differentiation, and activation of precursor cells and mature granulocytes and macrophages (11, 14). It is produced by several cell types (monocytes, T cells, fibroblasts and endothelial cells) including macrophages (20), and its synthesis is induced via the NF-κB transcriptional activator (30). GM-CSF also regulates the transcription and release of several other cytokines and the expression of major histocompatibility complex class II molecules and Fc receptors (7, 10). Many cytokines such as interleukin 1 (IL-1), IL-4, IL-10, tumor necrosis factor alpha, and gamma interferon regulate the expression of GM-CSF (9, 15, 18, 41). GM-CSF is involved in a wide range of immunological effects such as tumoricidal, microbial, and oxidative burst activities of both human and mouse macrophages (8, 13, 27). It is also a positive regulator of immune functions during bacterial and parasitic infections (3, 19, 26).

Trypanosoma cruzi, the etiological agent causing Chagas' disease, affects several million people in South and Central America. This parasitic protozoan infects several cells of the mammalian host and particularly macrophages, fibroblasts, and nerve and muscle cells (34). Various cytokines (IL-2, IL-4, IL-6, IL-10, tumor necrosis factor alpha, gamma interferon, and transforming growth factor beta) are involved in the course of the infection (1, 12, 17, 22, 23, 28, 31, 32, 35–37, 38, 42). However, the involvement of GM-CSF in *T. cruzi*-infected mice is still poorly understood, although this cytokine decreases the rate of infection of both nonactivated and gamma interferon-activated macrophages infected with *T. cruzi* in vitro (24), and in vivo it restores the deficient immune response of chronically infected mice (26).

The role of GM-CSF in *T. cruzi* infection was therefore emphasized by measuring the plasma GM-CSF concentration, parasitemia, and cumulative mortality in *T. cruzi*-infected mice during the acute and chronic phases of infection. The role of GM-CSF was assessed by injecting neutralizing anti-GM-CSF monoclonal antibodies (MAb) or recombinant mouse GM-CSF (rmGM-CSF) into *T. cruzi*-infected mice.

T. cruzi (Tehuantepec strain) was maintained by weekly intraperitoneal inoculations in BALB/c mice. To obtain large amount of parasites for both in vivo and in vitro experiments, trypomastigotes (2.5×10^5 per rat) were inoculated into 700-rad X-ray-irradiated F344 Fischer rats (Iffa Credo, Brussels, Belgium). Trypomastigotes were obtained from the blood (containing 10 U of heparin per ml) of infected rats by ion-exchange chromatography on DEAE-cellulose (Whatman DE 52) equilibrated with phosphate-saline-glucose buffer at pH 7.4. Trypomastigotes were centrifuged (15 min, $1,800 \times g$, 4°C) and resuspended in endotoxin-free phosphate-buffered saline (PBS) (25, 40).

Male BALB/c mice (6 to 8 weeks old) were purchased from Bantin & Kingman Universal (Hull, United Kingdom). The GM-CSF concentrations in the plasma of infected and uninfected mice were measured by enzyme-linked immunosorbent assay (ELISA) using rmGM-CSF as control (Endogen, Boston, Mass.). Anti-GM-CSF MAb (300 pg/ml) completely inhibited the detection of any GM-CSF in plasma samples from infected mice. The detection limit of the assay was 10 pg/ml. Changes in endogenous plasma GM-CSF concentration were monitored in 40 mice inoculated intraperitoneally with 50 trypomastigotes in 0.2 ml of PBS. A control group of 20 infected mice was injected with 0.2 ml of PBS and treated in the same way. Blood samples (25 μl per mouse) were taken at various times. They were obtained by tail incision and with heparinized microhematocrit tubes (Clay Adams, Parsippany, N.J.). Individual blood samples were pooled two by two and centrifuged (10 min, $800 \times g$), and plasma samples were harvested and stored at −70°C until use. Indirect immunofluorescence tests indicated that plasma samples from infected mice in the acute phase (day 22 postinfection [p.i.]) were weakly positive (anti-

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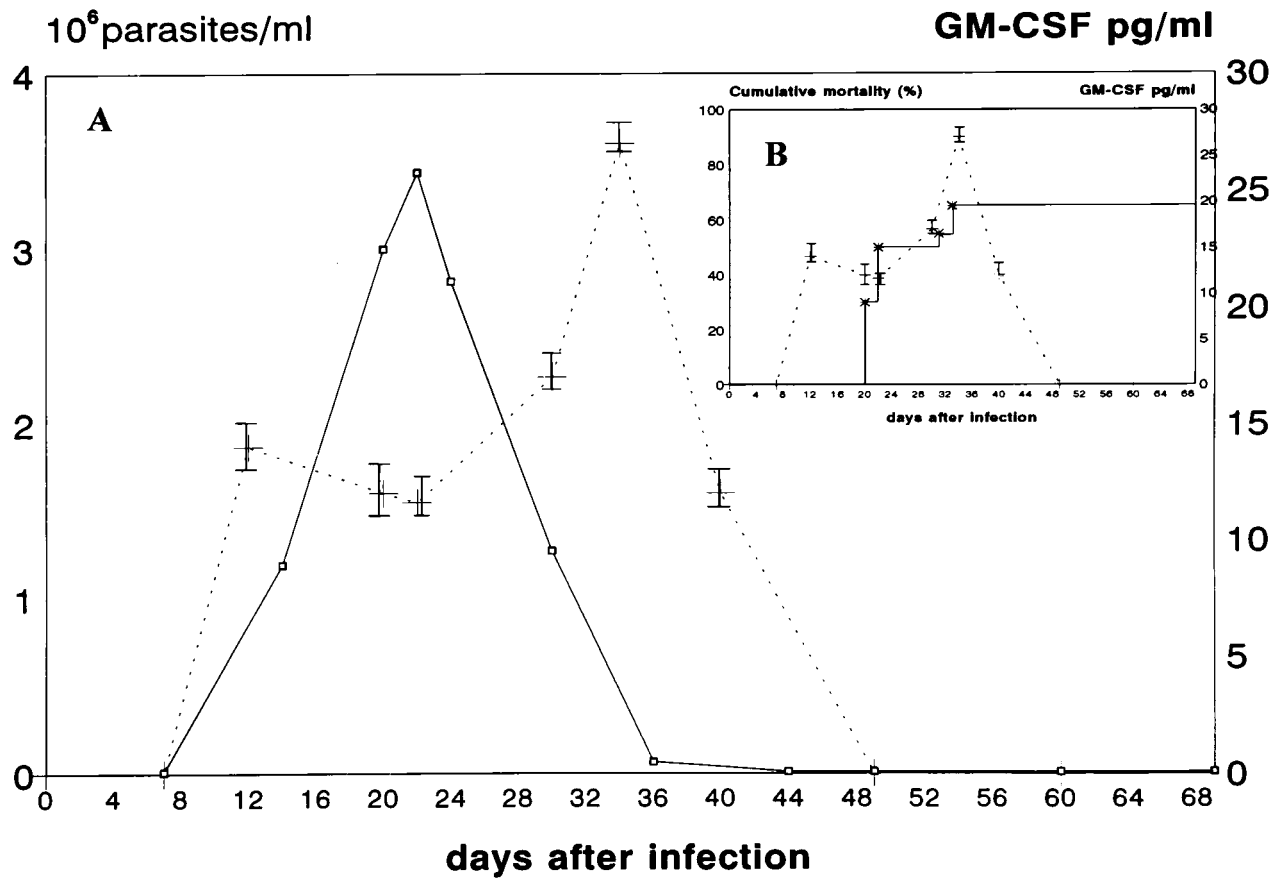

FIG. 1. Plasma GM-CSF concentration (dotted lines), parasitemia (solid line in panel A), and cumulative mortality (solid line in panel B) of mice infected with *T. cruzi*. Representative data from one of two independent experiments are shown. $n = 40$. Bars, standard errors of the means.

body titer, 1/100) for anti-*T. cruzi* antibodies while plasma samples from mice in the chronic phase (day 70 p.i.) were highly positive (antibody titer, 1/800). Parasitemia was monitored by counting trypomastigotes in blood samples collected by tail incision every 2 days, and survival rates were determined each day (5).

Ten *T. cruzi*-infected mice were given 200 μg of neutralizing anti-GM-CSF mouse MAb (immunoglobulin G2a; Endogen) on days 7 and 14 p.i. Two control groups of six infected mice were each injected intraperitoneally with an isotype-matched control MAb (200 μg per mouse; IR418; IMEX, Brussels, Belgium) or with PBS on the same days. The endotoxin concentrations of the anti-GM-CSF and IR418 MAb were below 15 pg/ml, as measured by the Endotoxin test (Chromogenix, Mälndal, Sweden).

The GM-CSF gene was isolated by PCR from the vector pCDNA I Amp ORF GM-CSF, a generous gift of J. C. Renaud (Brussels, Belgium). It was cloned as a *Bgl*II-*Hin*dIII fragment in the bacterial expression vector pQE 12 (Qiagen GmbH, Hilden, Germany). The resulting plasmid, pQE-moGMCSF, was transformed into M15 *Escherichia coli* cells. The expression of the recombinant protein was induced by the addition of isopropyl β-D-thiogalactoside (1 mM; Immunosource, Zoersel, Belgium). After 3 h of incubation, most of the rmGM-CSF was present in the bacteria as inclusion bodies, which were purified as previously described (6). Finally, the inclusion bodies were denatured and resuspended in 8 M urea–0.1 NaH_2PO_4–0.01 Tris (hydroxymethyl)-aminomethane (Sigma Chemical Co., St. Louis, Mo.) (pH 8.0). The crude, denatured protein mixture was diluted 1/100 in a refolding buffer (1 M urea, 50 mM Tris HCl, 0.005% Tween 20, 2 mM reduced glutathione, and 0.02 mM oxidized glutathione; pH 8.0; Sigma) and left at 4°C for 40 h. The refolded crude rmGM-CSF was loaded onto a MonoS fast-flow column (Pharmacia Biotech Benelux, Roosendaal, The Netherlands) in the refolding buffer. Pure rmGM-CSF was eluted as a sharp peak with an NaCl gradient in 50 mM Tris HCl, pH 8.0. The purity of the eluted protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, rmGM-CSF appeared on the gel as a discrete band of 16.5 kDa. The biological activity of the rmGM-CSF was assessed in a proliferation assay with the GM-CSF-dependent cell line NFS-60. Proliferation was measured by incorporation of H_3-labeled thymidine. rmGM-CSF was diluted in endotoxin-free PBS, aliquoted, and stored at −70°C until use. The endotoxin level in the purified rmGM-CSF preparation was less than 15 pg of protein per ml as measured by the Endotoxin test.

T. cruzi-infected and uninfected mice were injected intraperitoneally with 500 pg of rmGM-CSF in 0.2 ml of PBS, every 2 days, starting 1 day prior to infection and continuing until day 38 p.i. Control groups of 10 mice (infected and noninfected) were injected with 0.2 ml of PBS.

Mouse peritoneal macrophages (MPM) were harvested from BALB/c mice, treated with rmGM-CSF or with PBS, and infected with *T. cruzi* or left uninfected. Peritoneal lavages were performed with chilled Hanks' balanced salt solution

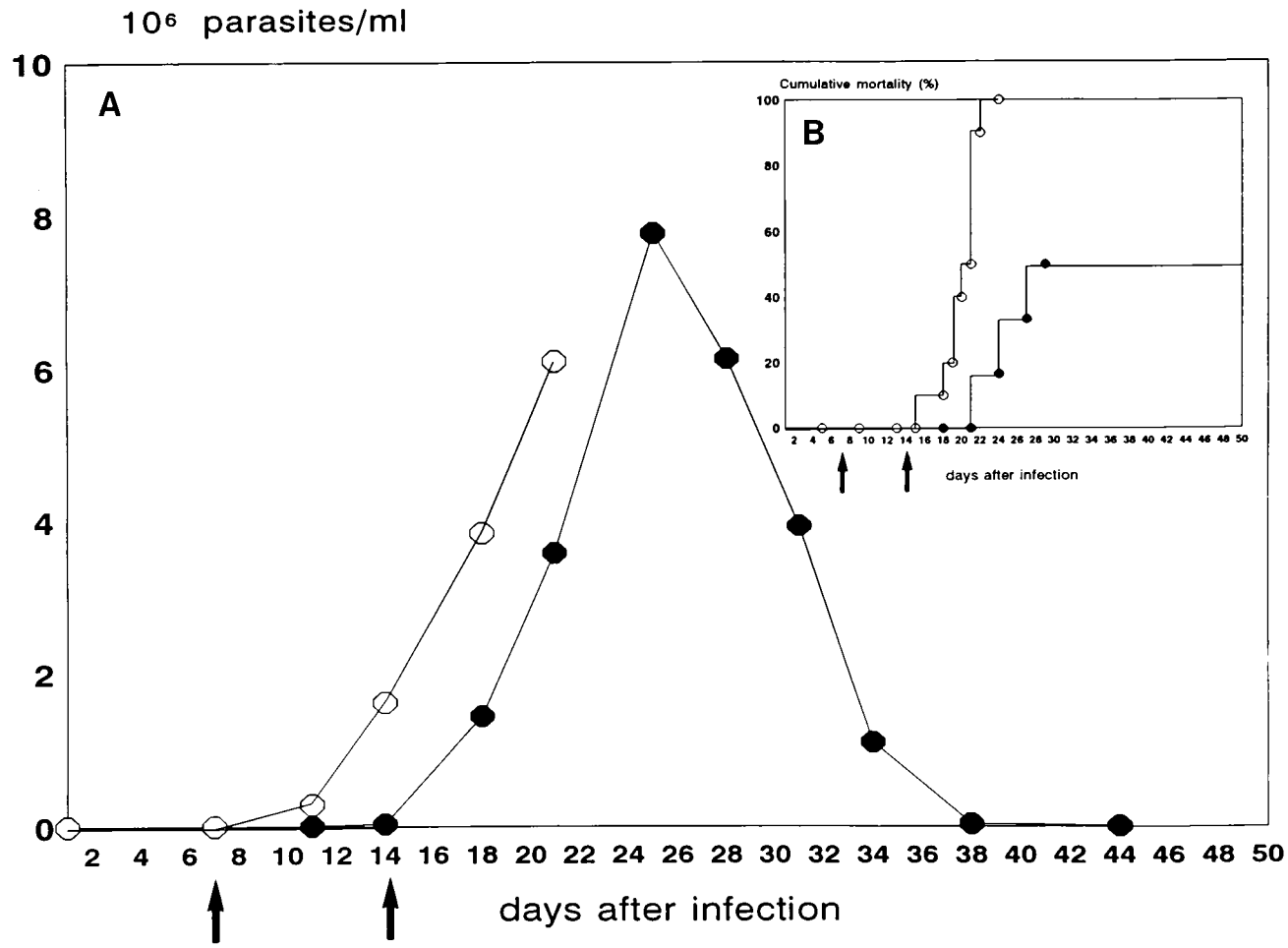

FIG. 2. Parasitemia and cumulative mortality in *T. cruzi*-infected mice treated with neutralizing anti-GM-CSF MAb. Mice were inoculated with *T. cruzi* trypomastigotes on day 0. Anti-GM-CSF MAb were injected on days 7 and 14 (arrows). The course of parasitemia (A) and cumulative mortality (B) were recorded for infected mice treated with anti-GM-CSF MAb (○) or with the isotype-matched control IR418 MAb (●). Representative data from one of two independent experiments are shown.

without Ca^{2+} and Mg^{2+} (pH 7.4) (GIBCO, Grand Island, N.Y.). After centrifugation (10 min, 400 × *g*), Hanks' balanced salt solution was removed and the samples were resuspended in RMPI 1640 medium supplemented with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; 25 mM), glutamine (2 mM), fetal calf serum (10%, mycoplasma free), penicillin (100 IU/ml), and streptomycin (100 μg/ml; GIBCO). MPM were allowed to adhere (10^5 cells per well) in eight-chamber Lab Teck slides (Nunc, Roskilde, Denmark) for 2 h at 37°C in a 5% CO_2 atmosphere. Nonadherent cells were removed by washing with prewarmed RPMI. MPM were incubated at 37°C in a 5% CO_2 atmosphere. Culture medium was renewed every 24 h for a total of 72 h. The endotoxin concentration was less than 27.5 pg/ml as measured by the Endotoxin test.

MPM were infected with *T. cruzi* by incubation at a parasite-to-cell ratio of 3:1 for 16 h. They were washed with warmed culture medium to remove free parasites. Forty-eight hours p.i., the MPM were washed, fixed with methanol, stained with Giemsa, and examined under the light microscope. The percentage of infected macrophages and the mean number of amastigotes per infected cell were recorded after examination of at least 200 cells per well.

The plasma GM-CSF concentration and parasitemia are shown in Fig. 1A. GM-CSF was undetectable at the beginning of infection, but its concentration increased sharply on day 12 p.i., while parasitemia became detectable on day 14 p.i. During the ascending phase of parasitemia from day 7 to 22 p.i., a leveling of the level of GM-CSF in plasma was observed while parasitemia peaked on day 22 p.i. The plasma GM-CSF increased further during the decreasing phase of parasitemia (days 22 to 36 p.i.) and peaked on day 34 p.i. It decreased thereafter, and GM-CSF became undetectable at day 49 p.i., 13 days after the disappearance of parasitemia (beginning of the chronic phase). However, surviving mice had a high plasma GM-CSF concentration and entered the chronic phase of infection. All the plasma samples from uninfected mice injected with PBS were negative for GM-CSF. Sixty-two and a half percent of infected mice died between days 0 and 33, before the circulating levels of GM-CSF reached a second peak. No mouse died after the second peak of plasma GM-CSF (Fig. 1B).

T. cruzi-infected mice were injected intraperitoneally with 200 μg of anti-GM-CSF MAb at days 7 and 14 p.i. Parasitemia was detected by day 11 and increased rapidly (Fig. 2A). All the mice died before day 24 p.i. In contrast, mice in the control

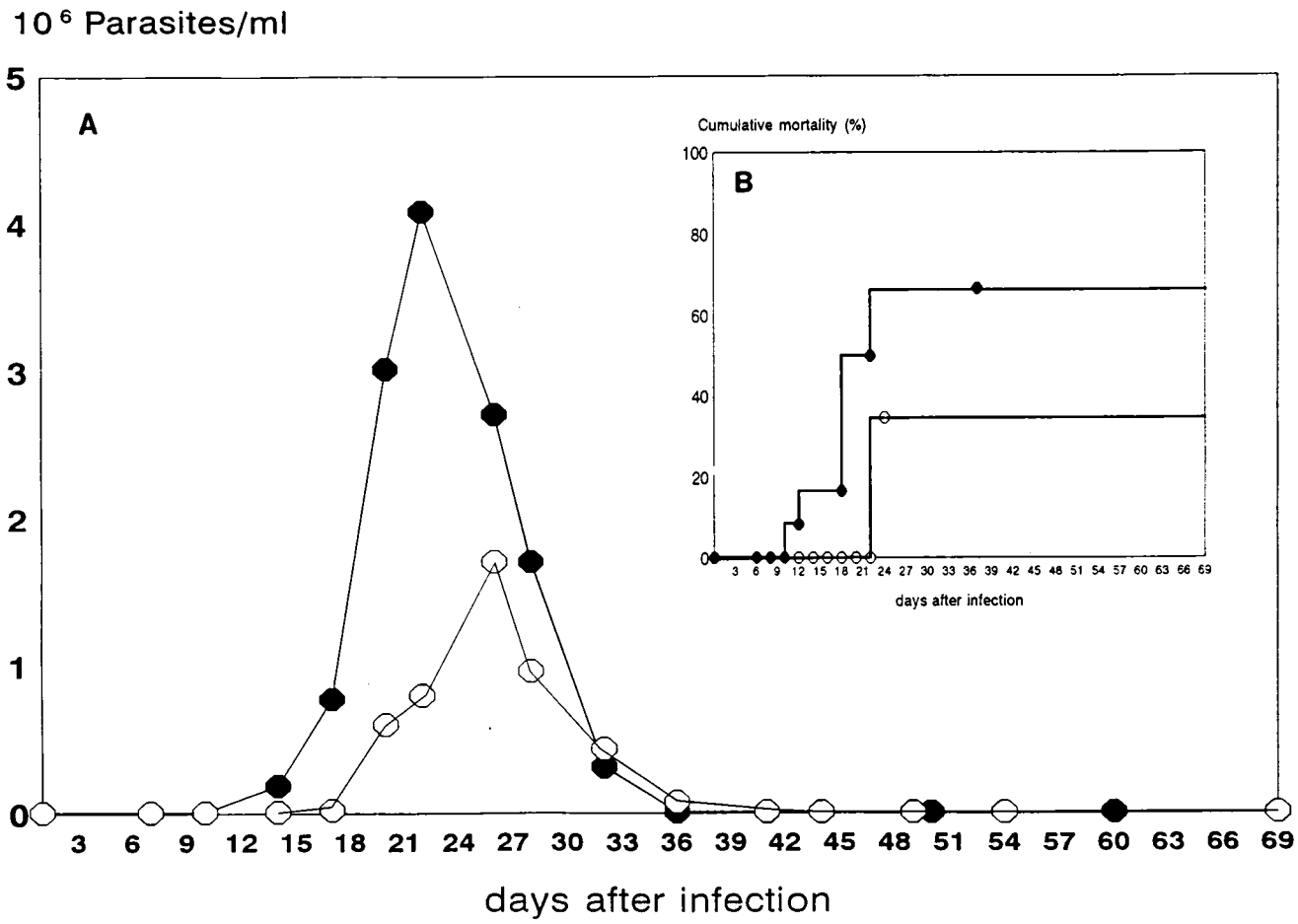

FIG. 3. Changes in parasitemia and cumulative mortality in *T. cruzi*-infected mice treated with rmGM-CSF. Mice were inoculated with *T. cruzi* trypomastigotes on day 0. GM-CSF injections began 1 day before infection and were repeated every 2 days until day 38. Parasitemia (A) and cumulative mortality (B) for infected mice treated with rmGM-CSF (○) or treated with PBS (●) were recorded. Representative data from one of two independent experiments are shown.

group injected with the isotype-matched control IR418 MAb survived, and parasitemia was detected only on day 18 p.i. (Fig. 2B). Similar results were obtained in the second control group injected with PBS (data not shown).

Injecting *T. cruzi*-infected mice with rmGM-CSF (500 pg per mouse every 2 days from one day before infection to day 38 p.i.) reduced the peak of parasitemia (2.6-fold decrease) (Fig. 3A). Furthermore, the peak of parasitemia was delayed and occurred on day 26 p.i. in rmGM-CSF-treated mice instead of on day 22 p.i. (control mice). Cumulative mortality (Fig. 3B) was also considerably reduced in rmGM-CSF-treated mice (35%) compared with control mice (68%). The first rmGM-CSF-treated mice died when parasitemia reached its maximum on day 26 p.i., whereas the first untreated mice died on day 10 p.i., when parasitemia was still undetectable.

MPM were harvested from PBS-treated or GM-CSF-treated and infected or uninfected mice on day 22 p.i. Some MPM from PBS-treated and infected mice were already infected (20.0 ± 4.3%), while MPM from GM-CSF-treated and infected mice were not infected. MPM were incubated ex vivo with *T. cruzi* trypomastigotes for 48 h. The percentages of infected MPM and the mean number of amastigotes per infected cell were determinated. Significantly fewer MPM from infected and GM-CSF-treated mice were infected than those from PBS-treated mice (Fig. 4). Similar results were obtained with MPM taken from uninfected and GM-CSF-treated mice. MPM from GM-CSF-treated and uninfected mice were also more readily infected than those harvested from GM-CSF-treated and infected mice.

The results demonstrate that endogenous GM-CSF has a protective action during the acute phase of *T. cruzi* infection in mice. The early production of endogenous GM-CSF, at the beginning of *T. cruzi* infection, is a part of the inflammatory response. The peak of parasitemia correlates with a slower increase in plasma GM-CSF. The peak of parasitemia results from the intense intracellular multiplication of the parasite and the disruption of many infected cells, particularly macrophages that are known to produce GM-CSF. In addition, there may be overproduction of IL-4, which can block GM-CSF synthesis, during the ascending phase of *T. cruzi* infection (15, 17, 29).

On the other hand, the plasma GM-CSF concentration is only partially reduced at the peak of infection. It could be sustained by several other types of GM-CSF-producing cells (Th1 and Th2 lymphocytes, mast cells, endothelial cells, and fibroblasts) that are not infected by *T. cruzi* or are not susceptible to the inhibiting effect of the parasite or parasite molecules. As a consequence, the rise in plasma GM-CSF is stopped, but the cytokine does not disappear totally from the plasma of infected mice.

Neutralization of endogenous GM-CSF increases both par-

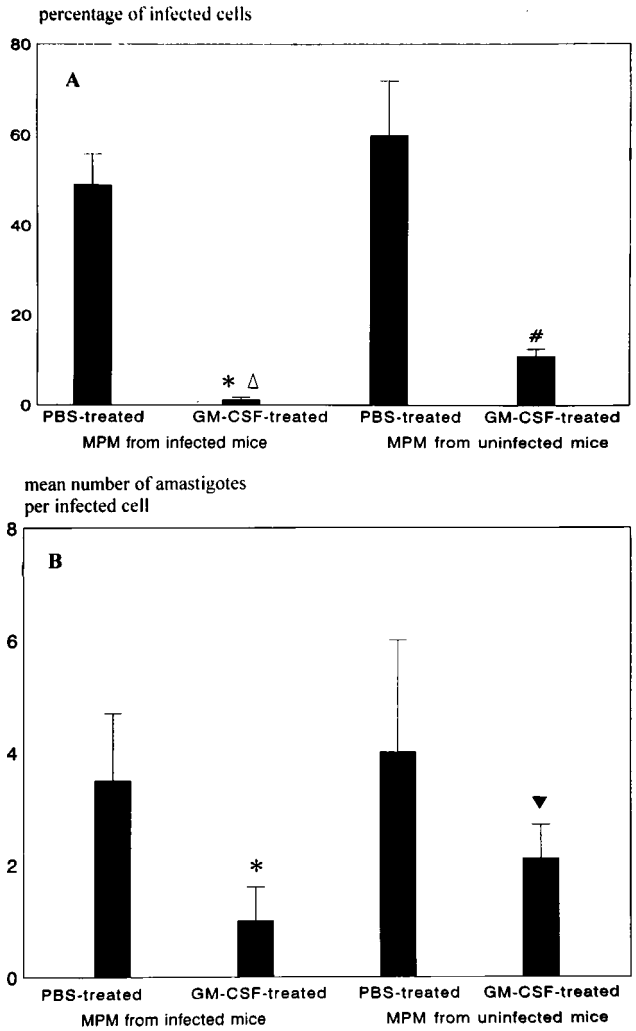

FIG. 4. Infection rate of macrophages harvested from rmGM-CSF- or PBS-treated mice infected (or not) with *T. cruzi* and incubated ex vivo with the parasite. MPM were harvested from GM-CSF- or PBS-treated mice and infected with *T. cruzi* or left uninfected. They were then incubated ex vivo with *T. cruzi*. The percentage of infected MPM (A) and the mean number of amastigotes per infected cell (B) were recorded after 2 days. *, $P < 0.01$ compared with infected and PBS-treated mice; # and ▼, $P < 0.01$ and $P < 0.02$, respectively, compared with uninfected and PBS-treated mice; △, $P < 0.01$ compared with uninfected and GM-CSF-treated mice (Student's *t* test). The data are means of two independent experiments with standard errors of the means.

asitemia and cumulative mortality. Trypomastigotes appeared very early in the blood of all neutralizing anti-GM-CSF-MAb-treated mice, and the mice died before the peak of parasitemia, probably because of a massive invasion of various organs. These data again indicate that GM-CSF can protect hosts in *T. cruzi* infection. They are also in line with the leveling of the concentration of GM-CSF in plasma induced by the parasite itself, likely to allow its rapid multiplication.

Exogenous rmGM-CSF decreases parasitemia and cumulative host mortality. In addition, mice which survived the acute phase of infection had high levels of GM-CSF in plasma at the beginning of the chronic phase and were still alive 70 days p.i. Indeed, GM-CSF appears to be a multipotent cytokine that can stimulate the immune system in various ways. First, this cytokine helps control *T. cruzi* infection during the chronic phase, stimulating IL-1 and IL-2 production and antigen presentation (26). Second, GM-CSF stimulates the humoral response, which also helps mice survive the acute phase (21, 33). Third, the end of the acute phase of *T. cruzi* infection also corresponds to that of the immunosuppressive phase, with inhibition of both IL-2 release and the mitogen-induced lymphoproliferative response (16, 28, 39). The high GM-CSF production at this time is reminiscent of the immunosuppressive activity of murine alveolar macrophages, which is inhibited by GM-CSF (4). Thus, the host's capacity to survive and to enter the chronic phase of infection could be linked to its capacity to produce enough GM-CSF, despite the leveling due to the infection.

MPM from GM-CSF-treated and uninfected mice are more infected than those from GM-CSF-treated and infected mice. This is particularly evident with MPM harvested from mice in the acute phase of infection. The reductions of both the percentage of infected cells and the mean number of amastigotes per infected cell suggest that MPM from infected mice treated with GM-CSF resist trypomastigote invasion. These data indicate also that the activation of MPM is not only the result of giving GM-CSF to infected mice. GM-CSF could act in synergy with other cytokines that activate macrophages during *T. cruzi* infection in vivo. GM-CSF could amplify the effects of endogenous gamma interferon produced during the acute phase of infection (37).

The results therefore emphasize the protective role of GM-CSF in experimental *T. cruzi* infection. Infection with *T. cruzi* first increases GM-CSF production, which is then impaired during the acute phase, when parasites are abundant in the blood. A second peak of GM-CSF occurs while parasitemia is decreasing and mice enter the chronic phase. Anti-GM-CSF MAb block the protective action of GM-CSF. Likely acting in synergy with other cytokines, exogenous GM-CSF decreases the severity of infection. These results indicate that, as suggested by recent work on leishmaniasis (2), it is worthwhile studying the use of GM-CSF in immunotherapy of Chagas' disease.

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