PREVENTION OF EXPERIMENTAL CEREBRAL MALARIA BY ANTICYTOKINE ANTIBODIES

Interleukin 3 and Granulocyte Macrophage Colony-stimulating Factor Are Intermediates in Increased Tumor Necrosis Factor Production and Macrophage Accumulation

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Recent studies have shown that TNF/cachectin, a cytokine mostly released by activated macrophages, plays a central role in experimental cerebral malaria (CM), an acute and lethal neurological syndrome that follows infection by *Plasmodium berghei* ANKA strain in mice (1). This experimental syndrome reproduces some of the features of human cerebral malaria, the most severe complication of *P. falciparum* infection in man. Experimental CM appears to represent a strictly T cell–dependent immunopathological complication of *P. berghei* infection, since the functional integrity of CD4$^+$ T lymphocytes is required for the neurological syndrome to occur (2). IL-3 and granulocyte/macrophage colony-stimulating factor (GM-CSF) are two cytokines released by activated T cells that stimulate the growth and differentiation of various hemopoietic cell lines, among which are macrophages (3–5). This work explores the possible role of these cytokines as intermediates in the marked TNF release leading to CM.

Materials and Methods

Mice. Female 8-wk-old CBA/Ca mice, originally obtained from Bomholtgard, Denmark, and bred in our animal facilities, were used throughout the experiments.

Infection. Malaria infection was initiated by asexual blood stages of the parasite, i.e., by intraperitoneal injection of 10^6 *P. berghei* ANKA (PbA)-infected erythrocytes, following the previously described procedure (2).

Anti-IL-3 and Anti-GM-CSF Antibodies. On day 3 of infection, mice received either anti–recombinant mouse IL-3 IgG (five mice), anti–recombinant mouse GM-CSF IgG (10 mice), or a combination of both antibodies (15 mice), and as controls, IgG fractions of the same rabbits after their depletion of anti-IL-3 and anti-rGM-CSF antibodies by affinity chromatography (see below) (10 mice). All antibody fractions were obtained through repetitive immunization of rabbits and were purified by affinity chromatography using purified rIL-3 (4) or rGM-CSF (6) (kindly provided by Drs. Geoff Schrimcher and John DeLamarter, Biogen, Cambridge, MA) coupled to Affigel 15 (Bio-Rad Laboratories, Richmond, CA), ultracentrifuged at 150,000 *g* for 150 min, and injected intravenously at the dose of 600 µg in 0.3 ml sterile PBS 0.01 M, pH 7.2. 1 µg of immuno-affinity-purified IgG or 10 µg of protein

A IgG fraction of the anti-IL-3 antibody were able to inhibit the activity of 2.7 U of native IL-3 or 10–30 U of rIL-3. 1 µg immuno-affinity-purified IgG or 10 µg of protein A IgG fraction of the anti-GM-CSF antibody were able to inhibit 10^3 U of recombinant GM-CSF. These determinations were performed using the cell lines described for the cytokine bioassays.

Hematological Studies. RBC counts were performed in an improved Neubauer hematocytometer (Assistent, Altnau, Switzerland) after a 1:1,000 dilution of retroorbital blood in PBS. Parasitemia levels were evaluated on Giemsa-stained smears.

Bioassays for Cytokines. Serum TNF activity was determined using the L929 bioassay (7). Serum IL-3 and GM-CSF activities were explored by studying serial dilutions of serum from infected animals for their ability to support growth of FDCP-1 (8) as described by Ihle et al. (9). Cell growth was colorimetrically estimated according to Mosmann (10).

Histopathological Studies. Mice presenting neurological signs after PbA infection were killed by cervical dislocation. Spleens were harvested and snap-frozen in liquid nitrogen and macrophage staining was revealed using histochemical staining for acid phosphatases.

Results and Discussion

Four groups of CBA/Ca mice, genetically susceptible to CM, were infected with PbA. 3 d later, these animals were treated with either rabbit anti–mouse rIL-3, anti–mouse rGM-CSF, a combination of both antibodies, or nonimmune rabbit IgG as controls. Fig. 1 shows the cumulative incidence of CM in these mice. While each antibody injected separately had no significant effect, the combined treatment dramatically reduced the incidence of the neurological syndrome (13%: two mice presenting CM among 15 treated mice). The protected mice had low or undetectable serum TNF levels (0.20 ± 0.13 ng/ml, n = 15), in contrast to what is observed in CM (4.98 ± 2.15 ng/ml, n = 6) (1). As was also observed in the case of prevention of CM by anti-TNF antibodies, the combined anti-rIL-3 anti-rGM-CSF antibody treatment had no effect on the progression of parasitaemia (data not shown). As a consequence of this beneficial effect on the development of CM in mice after *P. berghei* infection, the survival of mice receiving the dual treatment with anti-IL-3 and anti-GM-CSF was significantly prolonged. These mice died during the third week of infection, with severe anemia and overwhelming parasitaemia, but without neurological lesions (Fig. 2).

It had been previously noted that murine malaria is associated with an accumulation of macrophages in lymphoid organs (11) and that, in mice with CM, such macrophage infiltration was more important than that observed in malaria-infected mice without the neurological syndrome (1). Since injections of rIL-3 and rGM-CSF in the mouse lead to an increased monocytosis and accumulation of macrophages, notably in the peritoneal cavity (3, 4), the possibility that antibodies against these cytokines may decrease the macrophage infiltration observed in CM was explored. On spleen
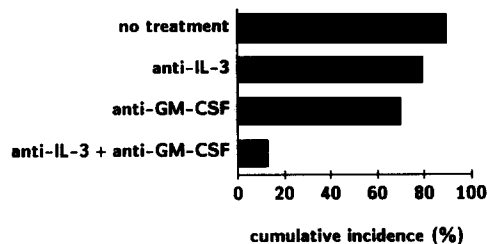

FIGURE 1. Cumulative incidence of the neurological syndrome (cerebral malaria, CM). Percentages of mice presenting clinical and histological evidence of neurological involvement were evaluated during PbA infection. Clinical signs occurred between day 7 and 10 of infection, and the most frequently observed were haemiparesis, paraparesis, ataxia, and convulsions. Histopathological lesions were focal intravascular accumulation of mononuclear cells and parasitized erythrocytes, as described (1).
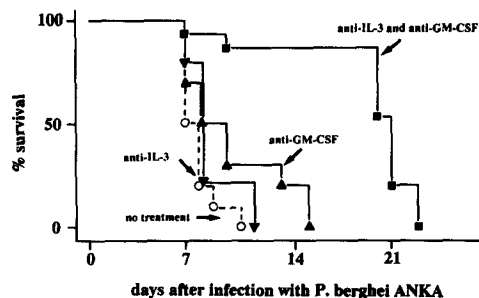
FIGURE 2.   Prolonged survival in PbA-infected CBA/Ca mice treated with both anti-IL-3 and anti-rGM-CSF antibodies. The acute phase of mortality, observed during the second week of infection by PbA, is due to the development of the neurological syndrome (CM), whereas the delayed mortality is due to severe anaemia ($<2.0 \times 10^6$ RBC/mm^3) and overwhelming parasitaemia ($>50\%$). Mice treated with the combination of anti-IL-3 and anti-GM-CSF antibodies survived until the third week, and died because of severe anaemia, without developing CM.

sections of mice with CM stained for acid phosphatases, it can be seen that macrophage infiltration is diffuse through the white and red pulp (Fig. 3 A). Spleen sections of mice protected by the double antibody treatment (Fig. 3 B) showed that, while macrophages in the red pulp were significantly more abundant than in normal, non infected mice (Fig. 3 C), the diffuse macrophage infiltration of the white pulp observed in mice developing CM was almost entirely prevented. Thus, it appears that an extensive macrophage accumulation resulting from the combined effects of IL-3 and GM-CSF (probably locally released during CM, since when using a bioassay (9), no activity was detectable in the serum) may be, at least in part, responsible for the high TNF blood level essential in the development of CM.

The data presented here suggest that IL-3 and GM-CSF are required for the increased in vivo TNF production associated with experimental CM. While the present and previous studies emphasize the roles played by CD4$^+$ T lymphocyte activation, macrophage accumulation, and release of IL-3, GM-CSF, and TNF in the occurrence of CM, other cell types and cytokines are likely to be involved in this complex pathogenic pathway. T lymphocytes are the only physiological source of IL-3 identified so far, but GM-CSF can also be produced, among other cells, by endothelial cells, in particular under stimulation by TNF (12, 13), suggesting the possibility of an autoamplifying mechanism. It has been proposed that endothelial cell alterations induced by TNF are involved in the pathogenesis of the neurovascular lesions typical of murine CM (1). Furthermore, the increased release of TNF in CM does not result simply from a quantitatively excessive accumulation of macrophages, as seen in the lymphoid organs. Stimulation of macrophages by IFN-γ, another cytokine released by activated T cells, is also required, as suggested by the observation that treatment PbA-infected mice with anti-IFN-γ antibodies prevent the increased TNF release and CM (Grau et al., manuscript in preparation). These data suggest that a cytokine cascade is involved in the pathogenesis of experimental CM. IFN-γ, among other actions, can activate macrophages and increase the TNF gene transcription (14), whereas IL-3 and GM-CSF act, at least in part, by increasing the accumulation of macrophages, thus contributing to the elevated serum level of TNF and its consequences. It should be mentioned that GM-CSF is also known to activate various macrophage functions (15) and that IL-3 and GM-CSF have been reported to lead to TNF release by human monocytes (16).

In man, neuropathological complications of malaria are the most common clinical presentation and cause of death in servere Plasmodium falciparum malaria (17–19).
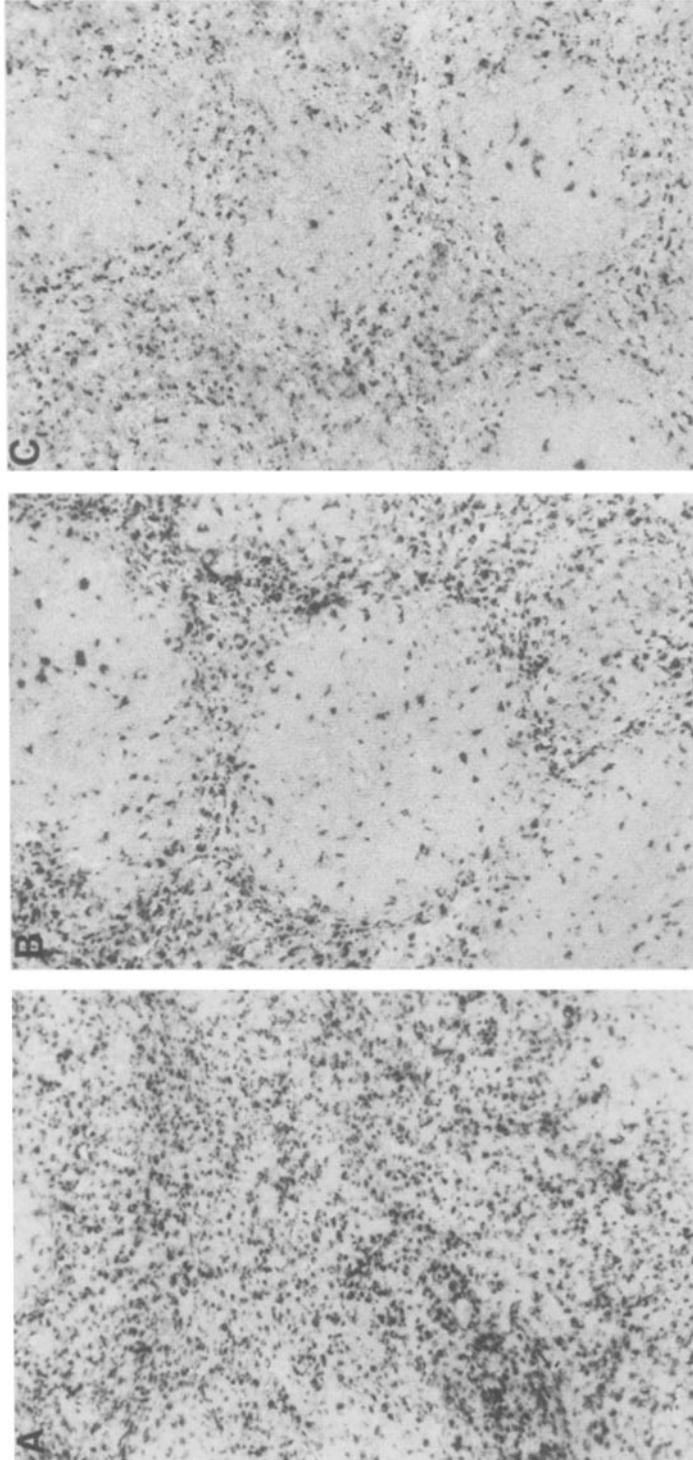
FIGURE 3.   Histologic pattern of spleen section stained for acid phosphatase. (A) Spleen of a mouse with CM (7 d after PbA infection). The diffuse macrophage infiltration does not allow to recognize white from red pulp. (B) Spleen of a mouse protected from CM by injection of both anti-IL-3 and anti-GM-CSF antibodies. The macrophage accumulation is markedly decreased and is limited to the red pulp, thus rendering the white pulp easily recognizable. (C) Spleen of an uninfected, age-matched CBA/Ca mouse used for comparison (× 400).

The precise pathogenic mechanisms of human CM and their possible resemblance to those of experimental mouse CM remain to be defined.

Summary

IL-3 and granulocyte/macrophage colony stimulating factor (GM-CSF) are two cytokines released by activated T lymphocytes that stimulate the growth and differentiation of various hematopoietic cell lines, among which are macrophages. It has been shown that TNF/cachectin, another cytokine that is released mostly by activated macrophages, plays a central role in experimental cerebral malaria (CM), an acute and lethal neurological syndrome induced by *Plasmodium berghei* ANKA infection in CBA mice. Since CM requires functional CD4$^+$ T lymphocytes to occur, we explored, by injecting rabbit antibodies to murine rIL-3 and/or GM-CSF, whether these cytokines are intermediates in the marked TNF release leading to CM. Treatment of infected mice with each antibody separately had no protective effect. In contrast, when both anti-rGM-CSF and anti-rIL-3 antibodies were injected together; (*a*) the occurrence of neurological syndrome was prevented in 90% of the cases; (*b*) the rise in serum TNF was prevented; and (*c*) macrophage accumulation in the spleen was significantly reduced. Murine CM appears to involve a cytokine cascade in which IL-3 and GM-CSF lead to the accumulation of TNF-releasing macrophages in vivo.

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