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Immunology

Monoclonal antibody against interferon γ can prevent experimental cerebral malaria and its associated overproduction of tumor necrosis factor

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ABSTRACT **Experimental cerebral malaria (ECM), a lethal hyperacute neurological syndrome associated with high blood levels of tumor necrosis factor, develops in genetically susceptible (CBA/Ca) mice 7 days after infection with *Plasmodium berghei* ANKA strain. Injections of neutralizing monoclonal antibody against recombinant murine interferon γ, not later than 4 days after infection, markedly reduced the incidence of ECM and the elevation in serum levels of tumor necrosis factor. This treatment prevented the cerebral lesions (plugging of brain vessels by monocytes, lymphocytes, and parasitized erythrocytes). In contrast, the extent of macrophage infiltration in lymphoid organs (which is a characteristic feature of mice developing ECM), as well as the course of infection, remained unaffected by the antibody treatment. Protected mice died at a later time of severe anemia and overwhelming parasitemia, the usual outcome of *P. berghei* infection in mice that are not susceptible to ECM. The present data indicate that interferon γ constitutes an important link in the cytokine network that leads to brain vessel inflammation in experimental malaria. It is proposed that interferon γ released by activated CD4+ T cells acts by augmenting both production and action of tumor necrosis factor.**

Cerebral malaria is the most severe complication occurring in malaria patients (1). A form of experimental cerebral malaria (ECM) that reproduces some features of the human disease develops as an acute lethal neurological syndrome in genetically susceptible CBA/Ca mice infected with *Plasmodium berghei* ANKA strain (PbA) (2). Several lines of evidence have allowed us to identify tumor necrosis factor (cachectin, TNF-α) as a crucial factor in the pathogenesis of ECM (3): (*i*) in PbA-infected CBA/Ca mice, blood TNF levels are elevated (from undetectable levels to >200 units/ml) at the time of occurrence of ECM; (*ii*) high blood TNF levels are not observed in the few genetically susceptible mice that do not develop ECM or in infected mice that are genetically resistant to ECM; (*iii*) passive immunization against TNF reduces the incidence of the syndrome (from 90% to <5%) and also prevents accumulation of mononuclear cells and parasitized erythrocytes in brain vessels, a lesion that is characteristically associated with ECM.

While it is likely that macrophages, which are found in strikingly increased numbers in the lymphoid organs of mice with ECM, are the major source of the high serum TNF levels, it has been observed that the presence of CD4+ T cells is also required for the complication to arise (2). The role of T cells might consist in releasing lymphokines that have direct or indirect inflammatory actions. Two such factors, granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3), both of which can be produced by activated T lymphocytes, have already been shown to be instrumental in ECM (4), since the mice simultaneously injected with antibodies against these two cytokines did not show macrophage accumulation, blood TNF increase, or ECM. The present study focused on the possible role of interferon γ (IFN-γ) the principal macrophage-activating factor released by T cells (5), which also induces in macrophages an increase in TNF mRNA transcription (6). The possibility that ECM could be modulated by treatment with anti-IFN-γ antibody was investigated.

MATERIALS AND METHODS

Mice. Female CBA/Ca mice, originally from Bomholtgard, Denmark, and bred in our animal facilities, were 8–10 weeks old at the time of infection.

Induction of ECM. The model system—CBA/Ca mice infected with PbA-parasitized erythrocytes—and the acute neurological syndrome developing subsequently have been described (2).

Antibodies. Rat anti-murine IFN-γ IgG2a monoclonal antibody (mAb) F3, derived from rat–mouse hybridomas, has a high binding potential and neutralizing activity (7). The batch of F3 used in the present study had a neutralizing titer of $10^{-5.25}$ to $10^{-5.5}$ against 10 units of IFN-γ. In most experiments the preparations consisted of ascitic fluid from hybridoma-bearing mice; 0.1 ml (10 neutralizing units) was injected per mouse. When indicated, a pure immunoglobulin preparation was used [purification on a column of Sepharose-bound mouse anti-rat κ-chain mAb, gift of H. Bazin (University of Leuven)]. Another rat anti-murine IFN-γ mAb, R4-6A2, derived from a rat–rat hybridoma (8), was kindly provided by G. Milon (Institute Pasteur, Paris); the titer of the ascitic fluid used was 10^{-4}. Rabbit polyclonal antibody was obtained by repeated immunization of rabbits with recombinant *Escherichia coli*-produced murine IFN-γ [from Genentech, gift of Günther Adolf (Boehringer, Vienna)] and was a protein A-purified IgG fraction. F1, another anti-murine IFN-γ IgG2a mAb, was used as control, as it is about 500-fold less potent than F3 in neutralizing the biological activities of IFN-γ. D9D10, a mouse IgG1 mAb directed against human IFN-γ (9), was also used as ascitic fluid as a control, since it does not crossreact with murine IFN-γ.

TNF Assay. Serum levels of TNF were determined by the L929 cell lytic assay (10) and expressed in units/ml; the number of units is defined as the reciprocal of the serum dilution leading to 50% lysis of the cells (3).

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Abbreviations: GM-CSF, granulocyte/macrophage colony-stimulating factor; IFN-γ, interferon γ; IL-3, interleukin 3; mAb, monoclonal antibody; PbA, *Plasmodium berghei* ANKA strain; TNF, tumor necrosis factor.

Histology. Spleens and lymph nodes from PbA-infected CBA/Ca mice were quick-frozen in liquid nitrogen. Macrophages were stained for alkaline phosphatase by standard procedures.

RESULTS

Effect of Anti-IFN-γ on ECM and the Course of Infection. Two groups of CBA/Ca mice were infected with PbA. Rabbit anti-IFN-γ polyclonal antibody or normal rabbit immunoglobulin (1 mg of IgG fraction) was given intravenously on day 4 of infection. ECM was observed in 7 of the 8 mice that received normal rabbit IgG, and only 2 out of 8 mice treated with the anti-IFN-γ developed the neurological syndrome and died. This suggested that anti-IFN-γ treatment might protect infected mice from ECM, and led to the use of a more powerful treatment, the intraperitoneal administration of the rat mAb F3 on days −4, 0, and +4 relative to the day of infection in groups of 10–12 CBA/Ca mice inoculated with PbA-parasitized erythrocytes. This time schedule of injections had been shown to be efficient in *in vivo* inflammation models (7, 11, 12). This treatment drastically reduced the incidence of ECM, from 87.5% in untreated controls to 10% in mice injected with F3 (Table 1). Another anti-murine IFN-γ mAb, R4-6A2, also provided a significant degree of protection. As controls, the rat anti-murine IFN-γ mAb F1 and the rat anti-human IFN-γ mAb D9D10, which do not neutralize murine IFN-γ, had no significant effect on the incidence of ECM (5 of 7 and 4 of 7 mice treated with F1 and D9D10, respectively, developed ECM). Untreated or control mAb-treated mice dying of ECM showed the classical clinical signs (i.e., hemi- or paraplegia, ataxia, coma, or convulsions) and 4 were autopsied. Histopathological analysis invariably revealed brain vascular plugging.

As a consequence of the reduced incidence of ECM, the survival time was prolonged in mice receiving antibodies that neutralized murine IFN-γ (Fig. 1). All mice died by the fourth week of infection, at which time they had severe anemia and overwhelming parasitemia but no neurological syndrome. The level of parasitemia was unaffected by F3 antibody treatment, despite its protective effect in ECM: the level of parasitemia was similar on day 21 in all surviving mice, whether treated with F3 antibody or control D9D10 antibody (Table 2). This failure of anti-IFN-γ antibodies to affect parasitemia was confirmed in an experiment of similar design in which mice of the same strain were infected with *Plasmodium yoelii*, which causes a nonlethal form of malaria in mice. In this model, anti-IFN-γ mAb likewise did not modify the development of infection or the course of convalescence (data not shown).

Time Requirements for Anti-IFN-γ Treatment. To determine the critical timing of anti-IFN-γ antibody treatment, a single dose of mAb F3 was given at various times relative to the time of parasite inoculation. Injection of F3 on day 4 was still effective in reducing the incidence of ECM, whereas injection on day 7, just before the first mice showed clinical signs of neurological defects, such as isolated palsies, or ataxia, had no protective effect (Table 3).

Table 1. Protective effect of IFN-γ-neutralizing mAbs against development of ECM in PbA-infected CBA/Ca mice

Exp.	ECM incidence*			
	Saline	F3	F3P	R4.6A2
1	9/12	0/10	—	3/10
2	10/10	0/10	3/7	1/7
3	9/10	3/10	0/8	—
Total	28/32 (87.5%)	3/30 (10%)†	3/15 (20%)†	4/17 (23.5%)†

Mice were given saline or the indicated mAb (0.1 ml, intraperitoneally) on days −4, 0, and +4 relative to the day of infection. F3 and R4.6A2 are mAbs that neutralize murine IFN-γ; F3P, immunoaffinity-purified F3. Titers are given in *Materials and Methods*.
*No. of mice developing lethal ECM/no. of mice in group.
†$P < 0.005$ (Fisher's exact test) vs. saline-treated animals.

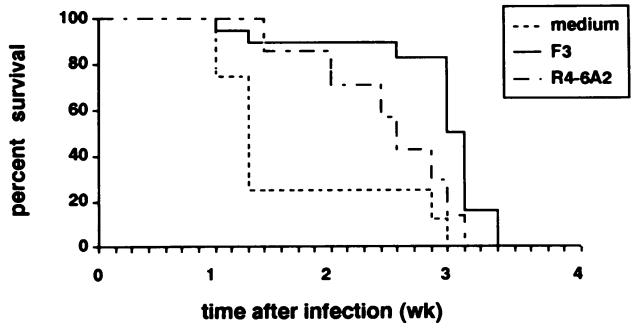

FIG. 1. Effect of anti-IFN-γ mAbs on the survival of PbA-infected CBA/Ca mice. Strongly neutralizing anti-IFN-γ mAbs, F3 and R4-6A2, significantly prolonged the survival of malaria-infected mice (n = 20 and 17, respectively), as compared with malaria-infected mice treated with culture medium as controls (n = 22). Nonneutralizing mAbs, F1 and D9D10, did not prolong survival: 7 mice treated with each of these mAbs died of ECM within the first 10 days of infection (data not shown).

Anti-IFN-γ Prevents the Rise in TNF Levels Associated with ECM. In view of the proven involvement of TNF-α in the pathogenesis of ECM, it was essential to study serum TNF levels in PbA-infected mice treated with anti-IFN-γ antibodies and protected against the brain disease. Serum TNF concentrations measured on day 7 of infection were markedly elevated in untreated mice showing signs of cerebral malaria (Table 4). Much lower levels were found in infected mice that escaped the neurological syndrome (pool of four series of experiments). In mice treated with mAb F3, TNF was undetectable in all but 1 of the 14 mice tested.

Effect of Anti-IFN-γ on Macrophage Accumulation. The brains and lymphoid organs of PbA-infected mice were examined 7 days after infection. The plugging of brain vessels by monocytes, lymphocytes, and parasitized erythrocytes was absent in anti-IFN-γ antibody-treated mice that did not develop ECM. However, the increased mononuclear cell infiltration in the spleen and lymph nodes that characteristically accompanies ECM (3, 4) was not prevented by anti-IFN-γ treatment.

DISCUSSION

The present study shows that *in vivo* neutralization of a single cytokine activity is able to prevent a complex neurological syndrome. We previously showed (2) that ECM is the expression of an immunopathological reaction, since $CD4^+$ T cells are necessary for the neurological syndrome to develop in PbA-infected mice of susceptible strains. Our present observations indicate that the contribution of T cells consists at least in part in producing IFN-γ. Production of IFN-γ, which

Table 2. Treatment with anti-IFN-γ mAbs does not affect the course of parasitemia in PbA-infected CBA/Ca mice

Day post-infection	% parasitized erythrocytes			
	Saline	F3	R4.6A2	D9D10
4	0.8 ± 0.7	0.7 ± 0.5	0.9 ± 0.6	0.9 ± 0.7
6	6.9 ± 1.1	6.2 ± 1.9	6.0 ± 1.8	5.9 ± 2.7
14	40.1 ± 5.12	34.3 ± 4.61	42.5 ± 5.25	40.0 ± 6.12
21	—	54.8 ± 4.71	51.5 ± 5.65	57.8 ± 7.91

Mice (n = 7 per group) were injected with saline or the indicated mAb on days −4, 0, and +4 relative to infection.

Table 3. Protective effect of a single injection of IFN-γ-neutralizing mAb F3 given at various times relative to infection

Day of F3 treatment	ECM incidence*
No F3	7/7
−4	2/7
0	1/7
+4	1/7
+7	7/7

*No. of mice developing lethal ECM/no. of mice in group.

is related to T-cell activation, has been described during murine malaria infection, and elevated levels of IFN-γ have been reported in the serum of patients with malaria (13, 14). It is evident from *in vitro* and *in vivo* studies that both IFN-γ and TNF can participate in the killing of malaria parasites inside erythrocytes (15). IFN-γ is able to reduce hepatocyte invasion by sporozoites (16) and schizogony (17), and this mechanism can be enhanced by vaccination (18). However, as judged from the lack of effect of anti-IFN-γ or anti-TNF antibody on the multiplication of parasites in mice that are protected from ECM, these cytokines do not have marked antiparasitic effects in the present experimental conditions.

Although IFN-γ has little or no direct inflammatory action, it is a powerful activator of macrophages, and it potentiates the effects of cytokines that do possess direct inflammation-promoting activities, in particular TNF-α and -β and interleukin 1, an effect observed both *in vitro* and *in vivo* (19, 20). In view of the high blood levels of TNF in ECM and the observation that this syndrome can be prevented by the administration of anti-TNF antibody, it was possible that the prevention of ECM by anti-IFN-γ treatment reflects the failure to potentiate TNF action by IFN-γ. However, it is apparent that IFN-γ acts first on TNF release, since serum TNF levels were dramatically reduced in mice treated with anti-IFN-γ antibody and protected against ECM, when compared to untreated mice that did develop ECM. Knowledge of the relative contributions of the cytokines might help to design new strategies for prevention or therapy of cerebral malaria in humans. These studies highlight the need to develop clinically compatible antibodies or other antagonists of certain cytokines.

From the present study on the involvement of IFN-γ in ECM and from a previous study showing that IL-3 and GM-CSF, other cytokines released by $CD4^+$ T cells, are also involved in the pathogenesis of ECM (4), a coherent picture of the roles of macrophages, T lymphocytes, and cytokines in the pathogenesis of ECM emerges. The requirement of $CD4^+$ T cells for the disease to develop (2) involves at least the release of IL-3, GM-CSF, and IFN-γ, since all these cytokines must be released in order to reach the high blood TNF level that appears to be eventually responsible for the occurrence of the acute cerebral vascular lesions (3). One aspect of the contributions of IL-3 and GM-CSF appears to consist in a vast increase in the number of mononuclear phagocytes, which is manifested by their accumulation in lymphoid organs (4). This probably represents the source of increased TNF production. One aspect of the contribution of IFN-γ is to enhance macrophage activation rather than accumulation. The increase in TNF mRNA transcription of macrophages by IFN-γ has indeed been documented (5, 6), and TNF has an upregulating effect on its own synthesis (21) as well as on the increase in TNF mRNA level triggered by IFN-γ (22), probably because it increases the number of IFN-γ receptors on cell surfaces (23). It seems reasonable to speculate that at some critical point, this cytokine cascade leads to a rapidly autoamplifying situation of TNF overproduction that ends in the cerebral vascular disaster responsible for ECM.

Table 4. Treatment with anti-IFN-γ mAb F3 prevents the increase in serum TNF in PbA-infected CBA/Ca mice

Treatment	*n*	Serum TNF, units/ml
None	15	0
PbA, without ECM*	14	6.4 ± 3.4
PbA, with ECM	19	211.6 ± 53.20
PbA plus mAb F3†	14	0.7 ± 2.5‡

TNF levels were measured 7 days after infection.
*PbA-infected mice that did not develop ECM were taken from eight different experiments.
†mAb F3 was given on days −4, 0, and +4 relative to infection; none of these mice developed ECM.
‡Thirteen mice had no detectable TNF; one had 10 units/ml.

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