Tumor Necrosis Factor (Cachectin) as an Essential Mediator in Murine Cerebral Malaria

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Tumor necrosis factor, or cachectin (TNF-α), a protein with a wide range of biological activities, is produced mainly by macrophages and may be important in inflammatory processes. The role of TNF-α in the pathogenesis of cerebral malaria was investigated in a murine model. Most CBA mice infected with *Plasmodium berghei* anka die between days 6 and 14 with acute neurological manifestations unrelated to the level of parasitemia, whereas mice of some other strains have malaria of the same severity that ends in death after 3 to 4 weeks without neurological manifestations. The activity of serum TNF-α was considerably increased in CBA/Ca mice with cerebral malaria but not in *Plasmodium berghei*–infected mice that did not develop this complication. One injection of rabbit antibody to TNF-α on day 4 or 7 fully protected infected mice from cerebral malaria without modifying the parasitemia, whereas immunoglobulins from normal rabbit had no effect. In mice with cerebral malaria, the cerebral vessels showed focal accumulations of packed macrophages often containing infected erythrocytes; this lesion was not seen in mice treated with antibody to TNF-α or in untreated mice without cerebral malaria. These findings indicate that TNF-α has an important role in the pathogenesis of cerebral malaria in this murine model and suggest that local accumulation and activation of macrophages may lead to the predominance of lesions in the central nervous system.

CEREBRAL MALARIA (CM) IS A SEVERE complication of *Plasmodium falciparum* infection, whose physiopathology is still poorly understood (*1, 2*). Although a minority (0.5 to 1%) of malaria patients develop CM, it is nevertheless commonly observed because of the high prevalence of this disease (up to 200 million cases per year). Sequestration of parasitized erythrocytes within the capillaries of the cerebral cortex may be one of the factors involved in this complication. Such sequestration may result from an interaction between endothelial cells and protrusions (knobs) of the parasitized erythrocytes mediated by specific receptors or by proteins that act as ligands, possibly as the result of endothelial cell damage, since areas of marked endothelial swelling are observed in the brain vessels (*3*).

The inoculation of mice with *P. berghei* anka (PbA) produces lesions that reproduce some features of human CM (*4*). In susceptible mouse strains (for example, CBA/Ca), CM is characterized by a neurologic syndrome appearing in about 80% of the mice 6 to 14 days after intraperitoneal injection of 10^6 infected erythrocytes, at a time of relatively low parasitemia of erythrocytes infected (5 to 10%) (*5*). Its manifestations include paralysis (mono-, hemi-, para-, or tetraplegia), deviation of the head, ataxia, convulsions, and a tendency to "roll over" in response to repeated stimuli: death ensues invariably within a few hours. Cerebral malaria does not occur in nude mice, and it was shown that T lymphocytes of the $L3T4^+$, helper phenotype are necessary for the development of this complication in susceptible strains, suggesting that a product released by stimulated $L3T4^+$ T lymphocytes is necessary for the development of cerebral lesions (*5*).

Several gross and histologic alterations of the brain have been described in murine CM, including hemorrhagic foci, most frequently in the meninges, cerebellum, and olfactory bulbs (*6*). Studies of "brain smears" and brain histologic sections revealed focal intravascular accumulations of large mononuclear cells, often containing malaria pigment or phagocytosed erythrocytes, in capillaries and venules. When mice with early signs of CM were injected intravenously with india ink and killed 1 hour later, intravascular macrophages containing carbon particles were readily visualized in about 30% of the capillaries on brain cortex smears (Fig. 1A). Ultrastructural signs of endothelial damage were also observed in these lesions (Fig. 1C), which appear to be characteristic of murine CM, since they were not seen in brain vessels of infected mice that did not display neurological signs of CM.

One of the mediators released in large amounts by activated macrophages is tumor necrosis factor, or cachectin (TNF-α) (*7*). In addition to its toxic effects on certain tumor cells and its inhibitory effect on the enzyme lipoprotein lipase, this protein induces several changes in endothelial cells, including an increased adhesiveness for polymorphonuclear leukocytes and, to a lesser extent, for monocytes (*8*). When injected in large amounts, TNF-α leads to a fatal state of shock accompanied by hemorrhage and necrosis of viscera, especially intestine, kidney, and lung (*9*). TNF-α also induces the synthesis of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 1 (IL-1) by endothelial cells and fibroblasts (*10*), and it is toxic for some parasites, including *Plasmodium* species (*11*).

To investigate the relation of CM to the

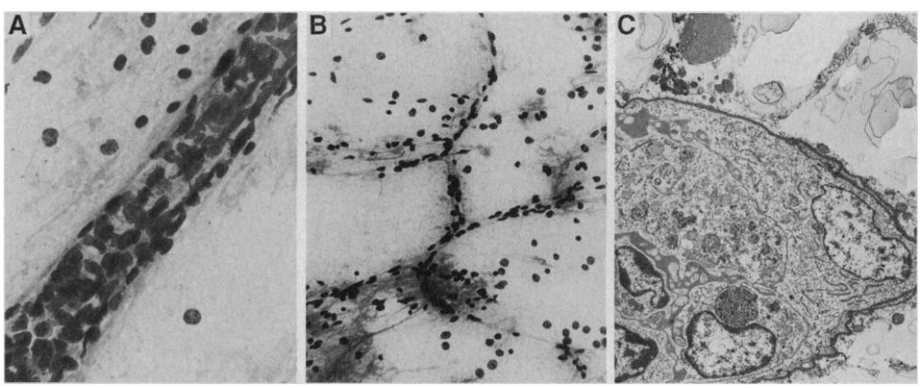

Fig. 1. (**A**) Intravascular accumulation of macrophages (with phagocytosed india ink) in capillaries from the brain cortex of a CBA/Ca mouse with CM, 7 days after infection with PbA (×160). This mouse was injected intravenously with india ink, 0.5 ml of a 1:10 dilution in phosphate buffered saline, as soon as neurological signs (left hemiplegia) appeared, and it was killed 1 hour later. The brain smear was prepared by smearing a 1-mm^3 fragment of brain cortex between two glass slides. (**B**) Brain cortex capillaries from a mouse protected against CM by treatment with anti-TNF-α IgG, prepared the same way, 1 hour after india ink injection. (**C**) Electron micrograph of an obstructed brain capillary of a CBA mouse with neurological signs (×3600). Two lymphocytes (lower left) are adjacent to a monocyte containing a large vacuole filled with carbon particles. Swollen endothelial cells, also containing small vacuoles with endocytosed ink, obstruct the lumen.

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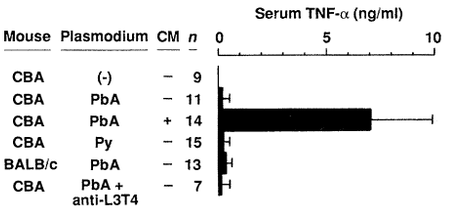

Fig. 2. Association between the occurrence of cerebral complications and high serum levels of TNF-α. Serum TNF-α levels were assayed in CBA/Ca mice (which develop CM) and in BALB/c mice (no CM) infected with 10^6 PbA-infected erythrocytes or 10^6 *P. yoelii*–infected erythrocytes. Blood samples were obtained from all mice as soon as neurological signs developed in those susceptible to CM. The sera of CBA mice without CM (n = 11) were obtained from mice that were infected but were not developing cerebral complications, and were pooled from five other series of experiments. For $L3T4^+$ T lymphocyte in vivo depletion, GK 1.5, a rat IgG2b monoclonal antibody to L3T4, was injected daily after infection with *P. berghei*, an injection schedule that completely prevents CM (*5*). Levels of TNF-α were determined by the L929 lytic assay (*12*) and expressed in relation to a reference standard preparation of recombinant murine TNF-α (3,471,000 U per milligram of protein, 1 unit being defined by the amount required to induce 50% lysis of L929 fibroblasts). Lytic activity was completely prevented in the presence of a rabbit antibody to mouse TNF-α. Bars indicate standard deviation. Symbols: minus sign, no development of CM; plus sign, occurrence of CM; minus sign in parentheses, no infection with parasite.

release of TNF-α by activated monocyte-macrophages, we assayed the serum levels of TNF-α in four groups of animals: uninfected CBA/Ca mice (genetically susceptible to CM), CBA/Ca mice infected with the CM-producing strain PbA or with the non-CM–producing strain of *P. yoelii*, and BALB/c mice infected with PbA, which did not develop CM. In all mice, blood was taken at the time when CM-susceptible mice displayed the first neurological signs of CM. Sera from normal mice did not contain detectable levels of TNF-α, whereas sera from all the infected mice showed a cytotoxic activity on the target L929 cells (*12*). This activity could be ascribed to TNF-α since it was totally inhibited in the presence of rabbit antiserum to *Escherichia coli*–recombinant mouse TNF-α. However, the serum concentrations of TNF-α in the mice with CM were markedly higher than those of the other groups of mice without CM (Fig. 2).

To explore further the pathogenesis of CM, we gave CBA/Ca mice a single injection (2 mg, intravenously) of an immunoglobulin G (IgG) fraction of either rabbit antiserum to mouse TNF-α or normal rabbit serum on day 4 or 7 after infection, that is, before the appearance of neurological signs. Among the mice treated with the antiserum to TNF-α, only 1 of 15 (6.6%) developed clinical signs of CM and died, whereas 15 of 19 (79%) of the mice untreated or treated with nonspecific IgG died of CM, in agreement with earlier observations (*5*) (Fig. 3, A and B). The protective effect of the antibody to TNF-α against CM lasted until the animals died with severe anemia. This treatment did not influence the parasitemia (Fig. 3C). In the sera of the treated animals, rabbit antiserum to TNF-α was still detectable by enzyme-linked immunosorbent assay (ELISA) up to 8 days after injection. When the injection of the antiserum to TNF-α was delayed until after the appearance of neurological signs, which precede death by a few hours, only one mouse out of three was protected against CM. The antiserum to TNF-α also prevented the focal macrophage accumulation in the brain vessels (Fig. 1B). Accumulations of macrophages in the spleen red pulp and in the lymph nodes of mice with CM were also decreased by treatment with antiserum to TNF-α.

The increased release of TNF-α during CM is likely to represent a response to lymphokines produced by stimulated $L3T4^+$ T lymphocytes, since depletion in $L3T4^+$ T cells induced in vivo by the injection of a monoclonal antibody to L3T4, which suppresses CM (*5*), also reduced the blood TNF-α levels (Fig. 2) and prevented the focal accumulation of macrophages in the brain vessels. In contrast to the effects of treatment with antibody to L3T4 (*13*), treatment with antibody to TNF-α did not influence the antibody response to *P. berghei* antigens. Therefore, its effects are unlikely to be due to a decreased immune response to the parasite. Some T lymphocyte products such as interleukin 3, GM-CSF, or γ-interferon are known to stimulate macrophage differentiation and TNF-α release by differentiated macrophages (*14*). One of the numerous effects of TNF-α is to induce endothelial changes, which include increased adhesiveness (*8*) and the synthesis of GM-CSF and IL-1 (*10*). Thus, high levels of TNF-α may create self-aggravating conditions, systemically by enhancing macrophage production and focally by enhancing endothelial alterations. The latter, by favoring monocyte accumulation, may lead to further local TNF-α and IL-1 release; such a process would become rapidly fatal when occurring in the brain vessels. Treatment with antibody to TNF-α may act by decreasing both macrophage proliferation, as suggested by the lesser accumulation of macrophages in the spleen and lymph nodes, and endothelial damage. Strain susceptibility to CM might reflect the capacity of macrophages from some mouse strains to produce larger amounts of TNF-α.

The detrimental role of TNF-α in murine CM may seem paradoxical in relation to the protective role for T lymphocytes and macrophages described in malarial infection. In response to parasite antigens, T lymphocytes can stimulate macrophages and lead to the release of such products as superoxide or TNF-α (*11*), which are potentially toxic for the parasites (*2*). The parasitic infection developing in our experimental conditions is probably too overwhelming to allow for the expression of such potentially protective mechanisms. It is a central theme in immunopathology that cells participating in the immune defense (for example, macrophages) and their products may have both beneficial and deleterious effects, depending on the degree of activation, its timing, and its location.

In conclusion, the release of TNF-α during malaria infection represents an important effector mechanism in the pathogenesis

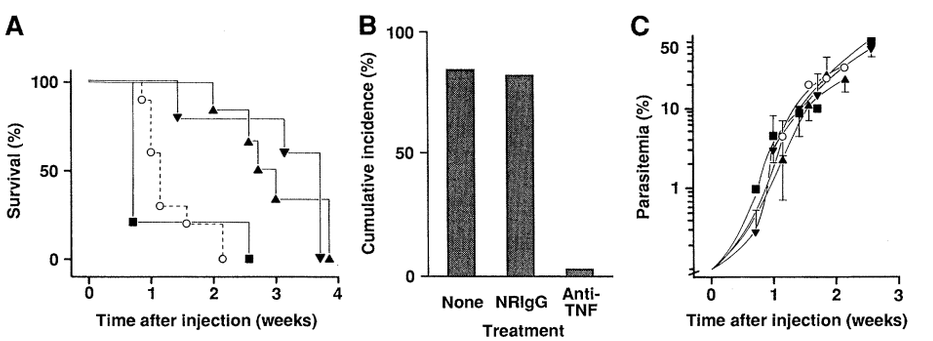

Fig. 3. Prevention of acute neurological complications in *P. berghei*–infected CBA/Ca mice treated with antibody to TNF-α. (**A**) survival, (**B**) cumulative incidence of the neurological syndrome, and (**C**) parasitemia (bars indicate standard deviation). Mice were injected intravenously with 2 mg of protein A (Pharmacia)–adsorbed rabbit antiserum to TNF-α or normal IgG in 0.5 ml of phosphate buffered saline, 0.01*M*, *p*H 7.2. This rabbit antibody was produced by immunization with purified, *Escherichia coli* recombinant mouse TNF-α in Freund's complete adjuvant (*16*). Seven mice were injected on day 7 (▲) and eight on day 4 (▼) with antibody to TNF-α. As a control, five mice were injected with normal rabbit IgG (NRIgG) on day 4 (■), and 14 mice received PbA alone (○). Similar treatment with IgG from rabbits injected only with Freund's complete adjuvant did not influence the course of CM.

of murine CM. It appears to be part of the cascade of pathogenic events probably initiated by products released by activated L3T4$^+$ T lymphocytes. This experimental model may not reproduce human CM, in which accumulations of packed infected erythrocytes but not of macrophages have been observed in cerebral vessels. However, both phenomena may result from a similar mechanism, such as an increased endothelial adhesiveness, which might reflect TNF-α–mediated vascular alterations. Therefore, TNF-α may also be of pathogenic significance in human cerebral malaria, a possibility supported by the observation of increased serum TNF-α in malaria patients (*15*).

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Neurosteroids: Cytochrome P-450$_{scc}$ in Rat Brain

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The steroid hormones corticosterone and testosterone are supplied to the central nervous system by endocrine glands, the adrenals and gonads. In contrast, the 3β-hydroxy-Δ5-derivatives of cholesterol, pregnenolone and dehydroepiandrosterone, accumulate in the rat brain through mechanisms independent of peripheral sources. Immunohistochemical studies have been performed with specific antibodies to bovine adrenal cytochrome P-450$_{scc}$, which is involved in cholesterol side-chain cleavage and pregnenolone formation. The enzyme was localized in the white matter throughout the brain. Scarce clusters of cell bodies were also stained in the entorhinal and cingulate cortex and in the olfactory bulb. These observations strongly support the existence of "neurosteroids," which have been posited on the basis of biochemical, physiological, and behavioral studies.

IN THE MALE ADULT RAT, CORTICOSTERONE and testosterone are secreted by endocrine glands, cross the blood-brain barrier, and are found in the central nervous system in concentrations lower than the plasma levels (*1*). Pregnenolone (Δ5-P) and dehydroepiandrosterone are 3β-hydroxy-Δ5-steroids, which derive from cholesterol by side-chain cleavage (*2*) and which are precursors of steroid hormones secreted by steroidogenic glandular cells. These 3β-hydroxy-Δ5-steroids accumulate in brain, in unconjugated, sulfate, and fatty ester forms, and through mechanisms at least partly independent of peripheral sources. Indeed, they persist after surgical or pharmacological elimination of adrenal and gonadal steroid secretion in rats and monkeys (*3*), and their concentration changes do not depend on peripheral glands in a variety of physiological situations, for example, over a 24-hour period (circadian rhythm) (*4*) and during ontogenetic development. In the olfactory bulb of male rats exposed to females, Δ5-P decreases, an effect that is dependent on testicular testosterone, whereas the female signal disappears after ovariectomy (*5*).

Early attempts to demonstrate the steroidogenic pathway from cholesterol in the brain, and particularly the biosynthesis of Δ5-P, were unsuccessful (*6*). We excluded the eventual role of brain storage derivatives and of extraglandular sources by injecting [^{3}H]Δ5-P subcutaneously and by way of an intracardiac or lateral ventricular routes in adult male rats: [^{3}H]Δ5-P was cleared from brain as rapidly as from plasma (*3, 7*). We were unable to demonstrate the biosynthesis of Δ5-P in brain, perhaps because the location of scarce steroid-producing cells was unknown. As an initial step in the demonstration of pregnenolone biosynthesis in brain, we looked for the presence of specific enzymes involved in cholesterol side-chain cleavage to Δ5-P (*2*). Cytochrome P-450$_{scc}$

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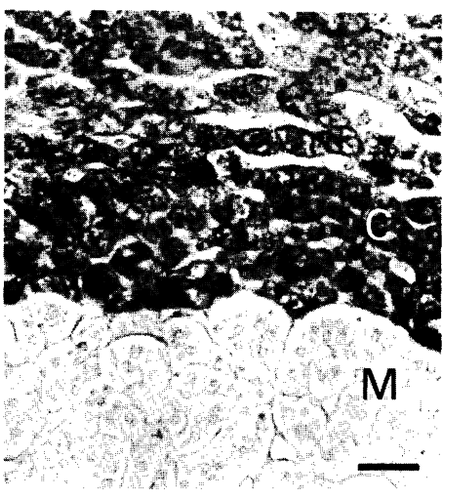

Fig. 1. Immunoperoxidase staining with cytochrome P-450$_{scc}$ antibodies from the adrenal gland of an adult female rat. For the immunohistochemical reaction, deparaffinized 7-μm-thick sections were rehydrated, rinsed in PBS, and incubated in 3% serum from a nonimmune goat. They were then incubated with the rabbit P-450$_{scc}$ antibodies (40 μg/ml, for 2 hours at room temperature). The biotinylated goat secondary antibody was added afterwards (dilution 1:200, 30 minutes), followed by the avidin-biotin-peroxidase complex (dilution 1:100, 30 minutes) (Vectastain reagents, Vector Laboratories). The peroxidase activity was revealed by 3,3′-diaminobenzidine tetrahydrochloride (0.5 mg/ml) in the presence of H_2O_2 (in tris buffer, *p*H 7.4). Sections were not counterstained. They were rinsed, dehydrated, and mounted. Controls were run on adjacent sections placed on the same slide. They included nonimmune rabbit immunoglobulin Gs, dilutions of specific antibody down to extinction of staining, and presaturation of P-450$_{scc}$ antibodies with purified antigen. Scale bar, 40 μm. Abbreviations, C, adrenal cortex (zona reticularis); M, adrenal medulla.