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IMMUNOPATHOLOGY OF MALARIA: ROLE OF CYTOKINE PRODUCTION AND ADHESION MOLECULES

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The pathological expression in malaria infection depends largely on immunopathologic responses induced by the parasite. In the past few years, we have attempted to analyze mechanisms by which inappropriate immune response to some malarial antigens can generate major complications of malaria and particularly neurovascular lesions. To this end, we have undertaken a study aimed at a more precise definition of immunopathological parameters of malaria infection, and more particularly those involved in cerebral malaria (CM). CM, the most severe complication of falciparum infection in man, represents a major problem of public health at the world level.

STUDIES IN EXPERIMENTAL CEREBRAL MALARIA

Interplay of TNF and other cytokines in CM

We have analyzed an experimental model of CM, the acute and lethal neurological syndrome induced by *P. berghei* ANKA (PbA) in mice (Grau et al., 1986). This model reproduces some but not all features of human CM, as recently reviewed (Lambert & Grau, 1986). We have shown that experimental CM is an immunopathological complication that is strictly dependent on the presence of CD4+ T cells (Grau et al., 1986). Also, the nature of the main lesion of CM, i.e. brain vessel plugging by leukocytes led us to explore the role of cytokines released by these cells. We have demonstrated that excessive release of tumor necrosis factor (TNF) plays a critical role in the pathogenesis of experimental CM. This contention is supported by three lines of evidence (Grau et al., 1987). First, markedly elevated serum TNF levels are seen only at the time of the neurological syndrome. Second, treatment of PbA-infected mice with a single injection of anti-mouse TNF antibody significantly protects against CM. This treatment prevents all pathological abnormalities including hemorrhages and the focal arrest of monocytes and other circulating leukocytes within brain capillaries and venules. In addition, the conspicuous macrophage accumulation in lymphoid organs is also suppressed by anti-TNF antibody treatment, suggesting that amplification loops of TNF on its own synthesis and on macrophage recruitment have been interrrupted by the antibody treatment. Third the administration of recombinant murine TNF to PbA infected of a CM-resistant strain induced a lethal neurological syndrome with all the clinical and histopathological features of CM (Grau et al., 1989a).

The localization of excessive TNF production during CM was analyzed by determining the expression of TNF mRNA in RNAs extracts from several organs. Using Northern blot analysis, we could show that in spleen and lungs, TNF mRNA accumulation was higher in mice developing CM than in mice infected to the same extent but without CM. Similarly, using polymerase chain reaction, we observed that intracerebral TNF mRNA accumulation is higher during CM than in uncomplicated malaria. The actual rate of transcription of TNF mRNA in these conditions is presently analyzed by run-on experiments. These data suggest that the following sequence of events is possibly leading to the triggering of CM: first, systemic TNF overproduction takes place in

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organs such as spleen and lungs; this oveproduction leads to detectable TNF levels in circulation. Second, at a certain point, blood TNF reaches a threshold level able to increase endothelial cell adhesiveness. Third, these changes of endothelial cells, occurring for unknown reasons mostly in brain and lungs, cause the focal arrest of leukocytes. Fourth, these locally sequestered leukocytes release themselves more TNF, which leads to auto-amplified cytotoxic effects on endothelial cells resulting in vascular wall damage and hemorrhagic necrosis. One can hypothesize that the functional heterogeneity of endothelial cells from various organs is one of the reasons why such lesions occur mainly in brain and lungs. This possibility is currently being analyzed.

Since the macrophage is the major source of TNF and since CD4+ T cells are required for CM to occur, we then analyzed the role of T-cell derived cytokines able to activate macrophages. In a series of experiments involving *in vivo* neutralization by anti-cytokine antibodies, we demonstrated that TNF hyperproduction is the result of a cytokine cascade. This cascade implicates IL-3 and GM-CSF, which act by enlarging the macrophage pool, as evident in lymphoid organs, and IFN-gamma, which upregulates macrophage functions, including the release of TNF (Grau et al., 1988, 1989b).

The possible implication of other mediators in CM was analyzed. Available data do not suggest that interleukin 1 or platelet-activating factor play a major role in the pathogenesis of CM (Grau et al., 1989a). The role of interleukin 6 (IL-6) was also evaluated (Grau et al., 1990). Our results suggest that large amounts of IL-6 are produced during PbA infection. However, since high serum IL-6 levels also occur in the absence of pathology and since anti-IL-6 monoclonal antibody treatment had no effect on CM, we concluded that this cytokine is not required for the development of CM. Interestingly, *in vivo* treatment by anti-IL-6 antibody prevented the rise in total IgG, but not IgM levels characteristic of malaria infection, without reducing the anti-malarial humoral response, therefore one has to hypothesize that cytokines other than IL-6 (such as IL-2 and IL-4) are more instrumental in specific antibody responses. IL-6 may thus be an important factor in the triggering of malarial-induced hypergammaglobulinemia, and the associated complications, such as glomerulonephritis or other immune-complex diseases (Grau et al., 1990). In addition, over-expression of IL-6 has been suggested in the development of B-cell lymphomas (Kawano et al., 1988). In malaria, IL-6 production might contribute to Burkitt's lymphomas, which have been associated to plasmodium infections in epidemiological studies (Epstein, 1984). The significance of IL-8 production in malaria may thus go beyond the cerebral syndrome: this cytokine might be involved in other aspects of malaria-induced pathological reactions.

Analysis of the relationship between cytokine production and susceptibility to CM

Differences in susceptibility to CM of various strains of mice – The development of CM was analyzed in ten inbred and one outbred strains of mice after infection with the standard inoculum of 10^6 PbA-infected red blood cells (Epstein et al., 1984). It was observed that while the degree of infection was essentially identical in all strains, there were marked differences in susceptibility to CM. Indeed, mice either developed CM in 60 to 95% of the cases (CBA/CA, CBA/HN, C57BL/6, SJL/J, DBA/2 and NMRI mice), or were totally resistant to this neurological complication and acute death (BALB/c, C3H/HeN, C3H/HeJ, DBA/2, and (NZBxNZW)F1 mice). Strains of mice found to be susceptible to CM were referred to as CM-S and the resistant ones as CM-R. It appeared that the genetic control of susceptibility to CM is not restricted to major histocompatibility (H-2) genes, since mice bearing the H-2^k haplotype were either susceptible (CBA) or resistant (C3H) to cerebral malaria.

Evaluation of the relationship between susceptibility to CM and TNF production – These mice of diverse genetic backgrounds were then studied for their capacity to produce TNF. The stimulant chosen was endotoxin (lipopolysaccharide, LPS), the most potent TNF inducer known so far (Beuter & Cerami, 1987). The production of TNF was assessed both *in vivo,* by measuring serum levels following injection of LPS into mice of various strains, and *in vitro,* by assaying supernatants, of peritoneal exudate cells (PEC) cultured in the presence of LPS (with or without IFN-gamma). The L929 bioassay was used to measure TNF activity in these experiments. Varying doses of *E. coli* O55:B5 LPS were used in both conditions, and sera or supernatants were collected at various times. Neither the level of TNF

production following stimulation by LPS nor the susceptibility to ECM are linked to the major histocompabitility complex. The two strains of mice most frequently studied in our laboratory, CBA (CM-S) and BALB/c (CM-R) mice did not differ significantly for their capacity to release TNF (30 mice tested) under LPS stimulation *in vivo* and *in vitro*. However, some trend can be suggested by our results: for instance, it was found that the highest TNF producers. SJL/J mice, are particularly susceptible to CM when infected with PbA. In addition, TNF concentration in the serum of these mice is dramatically elevated at the time of the neurological syndrome, as it had first been observed in CBA/Ca mice. Histological examination revealed brain vessel plugging by mononuclear cells and parasitized erythrocytes, which is characteristic of the lesion of CM in mouse.

In contrast, (NZBxNZW) F1 mice, which are the lowest TNF producers known so far, are resistant to the acute neurological syndrome. No TNF was detectable in their blood and no plugging in their brain vessels was observed during *P. berghei* infection. We also found that C3H/HeJ mice, carrying the lps-d mutation and thus unable to produce TNF upon stimulation by LPS, are resistant to CM. More experiments are in progress to critically evaluate the role of the TNF production capacity in CM, particularly in the presence of plasmodial extracts. Susceptibility to develop CM might also be due to abnormal release of other cytokines. The capacity of different strains of mice to produce diverse cytokines, such as IL-3, IFN-gamma and others, as discussed in the next paragraph.

Evaluation of T cell reactivity during Pba infection: proliferative capacities and cytokine release – First, we analyzed the proliferative response of T cells from PbA-infected mice to several PbA antigen extracts: living PbA-infected red blood cells (pRBC), frozen-thawed preparations, and soluble antigens released during a 24 h-culture of these infected erythrocytes. We selected lymph node cell populations because spleens during malaria infection have been shown to contain large numbers of erythropoietic cells, as a result of the extramedullary hematopoiesis (Grau et al., 1986). Marked differences in T cell proliferation was found: in the presence of pRBC, lymph node cells from infected CBA/Ca mice (CM-S) proliferated significantly more than those from infected BALB/c mice (CM-R) (5 individual mice tested, Mann-Whitney $p = 0.009$ for proliferations with 10^6 and 3.10^5 prBC/ml). In contrast, T cells from non-infected CBA/Ca and BALB/c mice did not show any difference in their constitutive response to either normal or infected red blood cells. While differences in T cell responses were noted in the presence of pRBC, no proliferation was seen in the presence of PbA soluble antigens, both from our laboratory and from that of Drs J. Taverne and J. H. L. Playfair, confirming that such soluble antigens are strictly T-cell independent. These preliminary observations might indicate that during PbA infection, T cells from mice of a CM-S strain display a more pronounced reactivity to malarial antigens than those from mice of a CM-R strain.

Secondly, we compared the capacity of cytokine production by lymph node cells from CBA/Ca and BALB/c mice, in response to PbA antigens. These preliminary data suggest that lymph node cells from PbA-infected CBA/Ca mice produced large amounts of IFN-gamma in response to pRBC, while those from PbA-infected BALB/c mice did dot. Responses to the mitogen, phytohemagglutinin (PHA) were identical in both strains. This finding might be consistent with the demonstration of the role of IFN-gamma in TNF overproduction and in CM (Grau et al., 1989b) and would suggest that CM is Th1-dependent pathology, i.e. a pathological reaction associated with a prominent activity of a CD4+ T cell subset with functional characteristics of Th1 subset (Mosmann et al., 1986). In contrast to IFN-gamma, the PbA-induced IL-3 production was higher in cultures of lymph node cells from infected BALB/c mice than in those from infected CBA/Ca mice. When considering constitutive production (i.e. by cells from non infected mice), IL-3 production was nonetheless higher in CBA/Ca than in BALB/c mice. The analysis of the ability to release IL-2, IL-4, IL-5, IL-6 and TNF under PbA antigen stimulation is underway. These preliminary results clearly deserve more investigations before we can draw any conclusion regarding the relationship between cytokine production profile and susceptibility to CM.

Studies on surface adhesion molecules in CM lesions

The main histological feature of these lesions is the focal arrest of macrophages and

lymphocytes in brain capillaries and venules, with ensuing damage of endothelial cells (EC) and hemorrhagic necrosis. Since expression of adhesion molecules can be upregulated by cytokines, and particularly TNF, we attempted to modulate vascular plugging by *in vivo* treatment with monoclonal antibodies (mAbs) directed to leukocyte adhesive membrane molecules, a group of heterodimers present on various types of cells. Anti-LFA-1 mAb given on day 6 of infection, i.e. the day before CM, delayed of 3 days the occurrence of neurological syndrome and death. But when given on days 6, 8, and 10, this mAb totally prevented CM. In contrast, anti-CR3 mAb had no protective effect against CM (Grau et al., 1991).

Blockade of LFA-1 might be protective in CM by preventing adherence of monocytes and lymphocytes to endothelial cells or alternatively by impairing T-cell activation. However, late administration of anti-CD4 mAb did not protect against CM, making it unlikely that anti-LFA-1 merely acts on T cells. These results suggest that LFA-1, but not CR3 adhesion molecules are important in the mechanisms of neurovascular lesions of experimental cerebral malaria. The ligand of LFA-1, the intercellular adhesion molecule 1 (ICAM-1) has been recently shown to be one of the surface molecule involved in the adherence of *P. falciparum*-infected erythrocytes to human umbilical vein endothelial cells (Berenolt et al., 1989). Since ICAM-1 expression can be upregulated by TNF (Rothlein et al., 1988) this finding might be relevant to high blood TNF levels found in patients with severe falciparum malaria, which are discussed in the next section.

STUDIES ON THE ROLE OF TNF AND OTHER CYTOKINES IN HUMAN CM

In our first study on TNF levels in Malawian children with severe malaria, in collaboration with T. E. Taylor, M. E. Molyneux and J. J. Wirima (Grau et al., 1989c) we have found that admission TNF levels exceeded those found at follow-up. High TNF levels were found to correlate with several parameters of severe malaria as previously defined (Taylor et al., 1989), including hypoglycemia, hyperparasitemia and age under three years. Also, circulating TNF was significantly higher in patients who died than in those who survived. The mortality rate increased with circulating TNF level. Because of this strong correlation with outcome, our data suggest that blood TNF concentrations on admission might have a prognostic value (Grau et al., 1989c).

A second study was performed in Zaire in collaboration with Nathan Shaffer, Phuc Nguyen-Dinh, and colleagues. The aim of this study was to further investigate the clinical features associated with elevated TNF concentrations. Eighty-seven children were enrolled. We found that plasma TNF concentrations were higher in 61 Zairian children with *P. falciparum* infection than in 26 severely ill, aparasitemic children ($p = 0.0003$). Among all parasitemic children, TNF levels increased with increasing levels of parasitemia ($p = 0.001$). On univariate analysis, elevated TNF levels were associated with hyperparasitemia, severe anemia, hypoglycemia, and young age, but not with cerebral malaria, HIV-1 seropositivity or fatal outcome. In this series, however, mortality rate was low (7%). TNF levels were elevated equally in children with cerebral malaria and in those with other signs of severe malaria. With multiple linear regression, TNF levels were found to be elevated independently in children with hyperparasitemia ($p = 0.001$) and severe anemia ($p = 0.04$). From this study, we concluded that high TNF levels are associated with several manifestations of severe falciparum malaria and are are not specific to cerebral malaria (Schaffer et al., 1991).

A third series of patients with falciparum malaria was studied in Malawi, in collaboration with Malcolm E. Molyneux, Terrie Taylor, and Jack J. Wirima. This study has also been carried out in the Department of Pediatrics at the Queen Elizabeth Central Hospital in Blantyre, Malawi, and criteria as well as methods are as described in the previous report. Circulating admission levels of cytokines (TNF, IL-1, and IFN-gamma) were studied in relation with several clinical and biological parameters. In addition, kinetics of cytokine changes after admission were followed up.

No correlation (using non-parametric Pearson's test) was found between admission levels of the three cytokines TNF, IL-1 and IFN-gamma. First, the relation with the severity of the neurological status was analyzed by studying cytokine levels in relation with the degree of neurological alterations. Coma score was assessed by the Blantyre modification of the Glasgow coma scale (Taylor et al., 1988). Using this system, a child with normal neurological status has a score of 5, whereas a to-

tally unresponsive child has a score of 0. Admission levels of TNF were significantly higher in patients with various degrees of neurological alterations (scores 0-4) than in children without any neurological impairment (score 5) (Mann-Whitney p = 0.0008). Moreover, there was a significant degree of correlation between blood TNF levels and the severity of the neurological syndrome: children with coma score 0-3 had higher TNF levels than those with coma score 4 and 5 (p = 0.023), although there was no difference between patients in deep coma (score 0) and those with mild neurological involvement (score 1-4). In contrast with TNF, neither admission blood levels of IL-1 nor these of IFN-gamma were associated with the neurological status.

Second, admission levels of the three cytokines were analyzed in relation to diverse clinical and biological parameters. TNF was found to correlate with a number of parameters, including blood glucose, blood lactate, time to become afebrile, time to become aparasitemic, degree of parasitemia and body temperature, but not with hematocrit, white blood cell counts nor with blood pH. This confirms and extends our observations in the first series of patients. In contrast, admission levels of IL-1 and IFN-gamma did not correlate with any of these parameters. The relation between TNF level and the time to become aparasitemic might be related to the fact that there was a strong correlation between this time and the degree of parasitemia (R = 0.382, $p < 0.001$). Indeed, as all patients received the same treatment (parenteral quinine), the parasite disapperance rate is likely to be constant. The relationship between cytokines and fever was particularly analyzed. While TNF (but not IL-1 and IFN-gamma) was found to correlate with body temperature, IFN-gamma (but not IL-1) levels were correlated with a temperature above 38.2 °C.

Sequential samples after admission were obtained in 46 patients. The study of blood cytokine kinetics showed essentially that high admission levels TNF and IFN-gamma dropped after 24 hr in all patients. In contrast, blood IL-1 concentrations were not changed on admission but rose significantly after 48 hr (Mann-Whitney p = 0.00001) and returned to normal levels at follow-up, i.e. when children were aparasitemic. Since the number of patients with sequelae or fatal outcome was very little, it was not possible to draw any conclusion yet as to whether the evolution of TNF levels might have been different in these patients, as it has recently been shown in patients with Gram-negative septic shock.

Current conclusions concerning human CM

A causal relationship between TNF and malaria pathology can be envisaged with respect to the symptomatology and the lesion of the disease. The essential pathologic feature of human CM consists of areas of hemorrhagic necrosis around brain venulae. It has been proposed that this is merely the mechanical result of the sequestration of parasitized red blood cells in small vessels, leading to obstruction of the microcirculation, and resulting in local hypoxia and hemorrhagic necrosis. *P. falciparum*-infected erythrocytes have been shown to adhere specifically to endothelial cells. A body of evidence suggests that this adherence occurs via specific interactions between several groups of surface proteins (reviewed in Howard & Gilladoga, 1989). If sequestration of parasitized red blood cells undoubtedly occurs, there are however several reasons to doubt that it can lead to hemorrhagic necrosis alone, i.e. in the absence of an effect of high concentrations of TNF. Sequestration of red blood cells correlates with parasitaemia, but there is no correlation between high parasitaemia and CM (Grau et al., 1989a). Thus, a hypothesis implying only alterations of the parasitized red cells would not explain why CM does not occur in all individuals with massive infection. In the mouse model, sequestration of parasitized is absent or marginal while hemorrhagic necrosis are fully developed, thus indicating that sequestration of red cells may not be necessary, but rather that of leukocytes and monocytes. TNF can not only increase the adhesiveness of endothelial cells for polymorphonuclear leukocytes, monocytes, lymphocytes and leukocyte cell lines (Pober, 1988). It also induces, upon infusion in mouse, morphological alterations of endothelial cell that are similar to those seen during CM (Piquet et al., 1990). This argues for a possibly crucial role of TNF in the triggering of sequestration. Therefore, in addition to anti-malarial therapy and attempts to interfere with adhesion, neutralization of TNF should be envisaged in the treatment of human CM. A clinical trial using a monoclonal antibody to human TNF has been initiated in Gambian children with severe falciparum malaria (Kwiatkouski et al., 1991).

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