INFECTION AND IMMUNITY, Apr. 1995, p. 1173–1175 Vol. 63, No. 4
0019-9567/95/$04.00+0

Strain Variation in Tumor Necrosis Factor Induction by Parasites from Children with Acute Falciparum Malaria

RICHARD J. ALLAN,[1] PAULINE BEATTIE,[1] CLIVE BATE,[1] MICHAEL BOELE VAN HENSBROEK,[2] STEVEN MORRIS-JONES,[2] BRIAN M. GREENWOOD,[2] AND DOMINIC KWIATKOWSKI[1]*

Department of Paediatrics, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom,[1] and Medical Research Council Laboratories, Fajara, The Gambia[2]

Received 8 August 1994/Returned for modification 26 September 1994/Accepted 6 January 1995

A small proportion of individuals infected with *Plasmodium falciparum* develop cerebral malaria. Why it affects some infected individuals but not others is poorly understood. Since tumor necrosis factor (TNF) has been implicated strongly in the pathogenesis of cerebral malaria, here we have compared different parasite isolates for their ability to induce TNF production by human mononuclear cells in vitro. Wild isolates were collected from 34 Gambian children with cerebral malaria and 66 children with uncomplicated malaria fever. Cerebral malaria isolates tended to stimulate more TNF production than mild malaria isolates, but there was considerable overlap between the two groups, and the present data provide only limited support for the hypothesis that cerebral malaria is caused by strains of *P. falciparum* inducing high levels of TNF. However, it is notable that the amounts of TNF induced by different wild isolates from a single locality differed by over 100-fold. The biological significance of this polymorphism deserves further scrutiny in view of the central role that TNF is believed to play in host defense and in the clinical symptomatology of human malaria.

Cerebral malaria (CM) is one of the most common causes of child death in sub-Saharan Africa, but it represents only a small proportion of all infections with *Plasmodium falciparum*. The critical factors that cause an infected child to develop CM remain obscure. It can occur at any level of parasitemia. Host genetic factors are believed to be involved (11), but they do not provide a full explanation. It is widely accepted that certain strains of parasite may be more likely than others to provoke CM, and yet there is little firm evidence as to what constitutes a virulent phenotype. Various parasite virulence factors have been proposed on the basis of experimental observations, but none has yet been validated in clinical investigations, with the exception of the rosetting phenotype; this phenotype has been found to be associated with CM in some studies (6) but not others (12).

At the cellular level, it is probable that at least two primary events underlie the pathology of CM: (i) the sequestration of schizonts in small cerebral blood vessels (16) and (ii) schizont rupture. Among the debris released by rupturing schizonts are toxins that stimulate monocytes and macrophages to produce tumor necrosis factor (TNF) and related cytokines (14). Circulating TNF levels are correlated with disease severity in childhood CM (10, 15). Experimental studies indicate that TNF may contribute to the pathogenesis of CM in several ways (7, 9). TNF produced in response to schizont rupture may exacerbate parasite sequestration by stimulating the endothelium to express parasite-binding receptors such as intercellular adhesion molecule-1 (5). It has also been proposed that TNF generated within the cerebral vasculature may lead to coma by stimulating the endothelium to release high levels of nitric oxide, which diffuses into the surrounding brain and disturbs neurotransmission (8).

These observations raise the question of whether the clinical severity of malaria could be related to the genetic propensity of the host to produce TNF, to the ability of the parasite to elicit this TNF response, or to both. Recent clinical data provide strong support for the first possibility, in that genetic variation in the TNF promoter region has been found to be associated with susceptibility to CM (17). The second possibility also merits investigation since cultured lines of *P. falciparum* have been shown to differ in their ability to induce TNF production by human monocytes (1). Here we report that wide variation in TNF-inducing activity can be observed among wild parasite isolates, and we examine the hypothesis that CM is associated with strains of *P. falciparum* that induce an abnormally high TNF response.

MATERIALS AND METHODS

This study was carried out at the Medical Research Council Laboratories, Fajara, The Gambia, and the Royal Victoria Hospital, Banjul, The Gambia, with the approval of the Gambian Government-Medical Research Council Joint Ethical Committee. At the time of maximal malaria transmission during two consecutive rainy seasons (October 1992 and October 1993), parasite isolates were collected from a total of 168 children who presented to the Medical Research Council Laboratories or the Royal Victoria Hospital with acute symptoms of malaria and asexual *P. falciparum* parasitemia. One hundred isolates survived in short-term culture (as defined below) and were entered into the study. Thirty-four of these children had CM, defined here as a Blantyre coma score of ≤ 2 with no evident cause of coma other than malaria. Sixty-six children had mild malaria (MM), defined as an uncomplicated febrile illness with no evident cause of fever other than malaria. The CM group had a median age of 3.0 years, and the MM group had a median age of 4.3 years. Seventeen (50%) of the CM group and 9 (14%) of the MM group reported having taken an antimalarial drug (usually chloroquine) in the 5 days before admission. The level of parasitemia on admission was slightly higher in the MM group (geometric mean, 4.9%; standard deviation [SD], 1.0 to 13.0%) than the CM group (geometric mean, 3.6%; SD, 2.3 to 10.3%), but this difference was not statistically significant.

All reagents and procedures were sterile and endotoxin-free. On admission, 0.5 ml of venous blood was collected in a sterile container with 5 ml of RPMI medium plus 10 U of preservative-free heparin per ml. It was kept at ambient temperature and processed within 4 h. Plasma was removed by centrifugation at $500 \times g$ for 15 min, and erythrocytes were washed three times in RPMI medium. After determining the starting level of parasitemia on a Giemsa-stained thin film, this was adjusted to 1% by adding erythrocytes from a healthy adult Gambian donor (blood group O; parasite negative). A second thin film was made after the erythrocytes had been added. Erythrocytes (3×10^8) were suspended in 2 ml of RPMI medium supplemented with 2 g of glucose per liter plus 20% of human serum (pooled from group AB^+ European donors and centrifuged at 100,000 $\times$

* Corresponding author. Phone: +44 865 221071. Fax: +44 865 220479. Electronic mail address: DOMINIC.KWIATKOWSKI@PAEDIATRICS.OX.AC.UK.

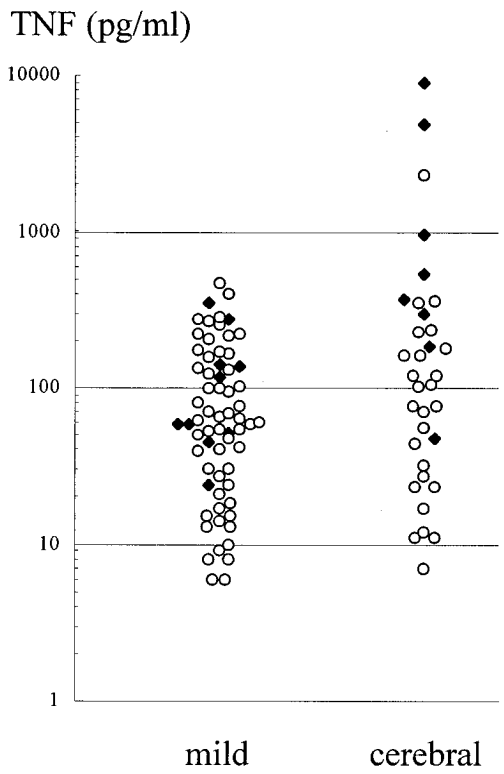

FIG. 1. Levels of TNF produced by human PBMC stimulated with lysed erythrocytic forms of *P. falciparum*. Isolates were diluted to 1% parasitemia and cultured for 48 h before lysis. Each point represents the mean value for a single isolate, tested in duplicate on PBMC from two donors. Samples collected in year 1 (◆) and year 2 (◯) are represented.

g for 18 h to remove sediment). This suspension was dispensed into an upright 15-cm^2 tissue culture flask and incubated at 37°C in 5% CO_2. After 48 h, a thin film was made to assess the parasitemia and specifically to determine whether the isolate had grown through one complete cycle in vitro. Our criterion was the appearance of new ring forms, indicating successful erythrocyte invasion. Thirty-four CM isolates and 66 MM isolates satisfied this criterion, and the remainder were excluded from the study.

Parasitized erythrocytes were harvested at 48 h and separated by centrifugation at 10,000 × *g* for 10 min, and the pellet was stored at −70°C. Within 1 month of collection, these samples were tested for their ability to induce TNF production by human peripheral blood mononuclear cells (PBMC). The methods have been described previously (1). In summary, venous blood was collected onto 10 U of heparin per ml, and PBMC were separated by density gradient centrifugation of Lymphoprep (Nycomed). After washing, PBMC were dispensed at 2×10^5 cells per well into sterile flat-bottom microtiter plates with 200 μl of minimal essential medium plus 1% heat-treated AB^+ European serum. Parasite isolates were lysed by one freeze-thaw cycle at −70°C and by mixing with 4 volumes of sterile pyrogen-free water. This lysate was added to PBMC at a final concentration of 1:20. After incubation at 37°C in 5% CO_2 for 18 h, PBMC supernatants were harvested and TNF concentrations were determined by enzyme-linked immunosorbent assay (15).

Since the samples were collected over two rainy seasons, we used the following strategy. We determined that the TNF-inducing activity in parasite lysates is stable for at least a month at −70°C. All of the samples collected during a single rainy season were tested on PBMC as a simultaneous batch. This was done within 1 month of collection in duplicate experiments using two separate mononuclear cell donors whose results were averaged. Since this meant that the samples from the 2 years of study were tested in two separate batches, we have treated this as a potential confounding factor in analyzing the data.

RESULTS

As shown in Fig. 1, the TNF responses to different wild isolates differed by over 100-fold. During the first malaria season, TNF levels ranged between 24 and 9,035 pg/ml, and during the second season, they ranged between 6 and 2,297 pg/ml.

In comparing the MM and CM isolates, we initially analyzed each year of the study separately, since there were inevitable sources of variation between the 2 years (see Discussion). During the first year, MM isolates gave a geometric mean TNF stimulation of 91 pg/ml ($n = 10$; 95% confidence interval [CI], 54 to 154) while CM isolates had a mean of 618 pg/ml ($n=8$; 95% CI, 188 to 2,033). In the second year, the mean was 56 pg/ml for MM isolates ($n = 56$; 95% CI, 41 to 76), and for CM isolates, it was 70 pg/ml ($n = 26$; 95% CI, 42 to 117). That is, CM isolates gave much higher TNF stimulation than MM isolates in the first year of the study ($P < 0.005$ by one-tailed *t* test), whereas during the second year, there was a weak trend in the same direction. If the results of the 2 years are pooled and subjected to an analysis of variance, with the year of sample collection included in the analysis as a covariate, then the overall trend is at the margins of statistical significance ($P = 0.04$).

Since prior antimalarial therapy is much more common in CM than in MM patients, we sought to exclude this as a potential confounding factor by analyzing only those isolates which showed clear evidence of reinvasion after 48 h in culture. The differences in TNF induction could not be attributed to variation in parasite stage, parasite density, or growth rates in culture. All isolates were adjusted to parasitemia levels of around 1% before culture was commenced. These levels were checked by thin film and found to be similar overall in both groups during both years of the study (CM geometric mean, 1.0% [SD, 0.6 to 1.7%]; MM geometric mean, 1.1% [SD, 0.7 to 1.8%]). When cultures were harvested at 48 h, the levels of parasitemia were similar in both groups (CM mean, 1.1% [SD, 0.6 to 2.1%] MM mean, 1.4% [SD, 0.7 to 2.5]). We observed no correlation between the logarithmic TNF response and the parasite growth rate in culture ($r = -0.03$, $P = 0.75$) or the absolute number of ring parasites when the culture was harvested at 48 h ($r = 0.03$, $P = 0.76$).

DISCUSSION

Although it is generally believed that parasite strain variation plays a major role in determining the clinical severity of falciparum malaria, specific virulence factors have proved difficult to identify. The present data show that there is wide variation in the TNF inducing activity of wild isolates of *P. falciparum* collected within a single locality from children with acute symptoms of malaria. Comparison of parasite isolates from children with different levels of disease severity provides limited support for the hypothesis that strains inducing high levels of TNF tend to predispose to CM. To interpret these findings, it is worth considering the technical and biological factors that tend to confound a phenotypic study of this sort.

It appears likely that we are observing a true phenotypic property of the parasite rather than some form of experimental variation since we have previously shown that laboratory isolates of *P. falciparum* maintain reproducible differences in their TNF-inducing activity when sampled repeatedly over a period of several weeks (1). In this project, we studied fresh wild isolates during their first growth cycle in vitro to avoid the possibility that TNF-inducing activity might be affected by adaptation to culture conditions. A disadvantage of using wild isolates is their variable viability, but we specifically selected isolates that showed clear evidence of reinvasion in vitro when the slides were inspected at 48 h. We cannot fully exclude the possibility that TNF-inducing activity might have been affected in vivo by antimalarial treatment. Prior chloroquine treatment was considerably more common in CM than MM patients and probably accounts for the somewhat lower levels of para-

sitemia in the CM patients on admission. However, TNF inducing activity was unrelated to the parasite growth rate in culture, indicating that chloroquine was unlikely to be a significant confounding factor. It is also conceivable that the ability of the parasite to induce TNF is influenced by intrinsic host factors and particularly by the acute inflammatory response. While this is entirely speculative, it cannot be ruled out as a potential contributor to the differences we have observed.

Levels of TNF produced in vitro depend on the responsiveness of mononuclear cells, which fluctuates from day to day. To minimize this source of experimental error, parasite lysates were pooled and tested in two batches, one at the end of each malaria season. In both years, it was apparent that wild isolates differed widely in their ability to induce TNF. However, the difference between CM and MM isolates, which was very marked in the first year, appeared as only a minor trend in the second year. This cannot be explained by any change in experimental procedure between the 2 years, but there are inevitable sources of variation in this sort of bioassay. These included the batches of medium and uninfected erythrocytes used in the parasite cultures, which differed between the 2 years, as well as the independent PBMC stimulation assays. We sought to correct for such confounders by stratifying for year in an analysis of variance using the whole data set, and this showed a marginally significant difference between the CM and MM isolates. This weak overall effect should be interpreted with caution, but the trend is suggestive. Although these data go against the idea that strains inducing high levels of TNF are the primary cause of CM, they are consistent with it being a predisposing factor, and further clinical investigation is warranted.

In trying to assess the pathological significance of strain variation, a crucial question is whether the parasites obtained from peripheral blood provide an accurate reflection of the subpopulation of parasites sequestered in the brain. This is true of phenotypic comparisons in general, but it is particularly relevant to TNF-inducing activity since current models of CM pathogenesis emphasize the importance of local cytokine production within cerebral blood vessels rather than systemic effects (5, 8, 9, 13). We have shown previously that, like the cytoadherence and rosetting phenotypes, the capacity of parasites to induce TNF is heterogeneous within a single wild isolate (1). The considerable degree of diversity that may exist within a single parasite strain raises the question of whether young parasites (which do not sequester) undergo random mixing when they are released from sites of sequestration. If parasites inducing high levels of TNF were more likely to be trapped at sites of sequestration, then the true effect of this phenotype on the pathogenesis of CM might be much stronger than our present results suggest.

CM and other life-threatening complications of *P. falciparum* infection are relatively rare events compared with the huge incidence of uncomplicated infection in residents of areas where malaria is endemic. In considering the dangers of excessive TNF production, it is important to recognize that this cytokine plays a central role in host defense against the parasite (7) and that the risks and benefits of the host TNF response are likely to have been factors in the evolution of a successful host-parasite relationship (13). To explore biological issues of this sort, there is a need for a better understanding of the molecular basis of TNF induction by the parasite. A series of studies by Playfair's group indicate that *Plasmodium* spp. have a major TNF toxin whose activity depends on a phosphatidylinositol-like structure (3, 4), and there is evidence that the critical element may be a form of glycosylphosphatidylinositol anchor (18). Recent work shows that a murine monoclonal antibody which recognizes phosphatidylinositol substantially inhibits the TNF induction by a wide variety of *P. falciparum* strains that differ in their TNF-inducing activity (2). These observations suggest that strain differences in TNF induction are due to variation in the abundance or the partial structure of a single toxin or family of toxins. In view of the central position that TNF occupies in both protective and pathological aspects of human malaria, the precise mechanism of this variation and its clinical significance represent intriguing biological questions.

ACKNOWLEDGMENTS

We thank Ayo Palmer, Veronia McGuire, and Sarah Meissner for their help in obtaining parasite isolates.

D. K. is a Medical Research Council Senior Clinical Fellow.

REFERENCES

1. **Allan, R. J., A. Rowe, and D. Kwiatkowski.** 1993. *Plasmodium falciparum* varies in its ability to induce tumor necrosis factor. Infect. Immun. **61:**4772–4776.
2. **Bate, C. A. W., and D. Kwiatkowski.** 1994. A monoclonal antibody that recognizes phosphatidylinositol inhibits TNF stimulation by different strains of *Plasmodium falciparum*. Infect. Immun. **62:**5261–5266.
3. **Bate, C. A. W., J. Taverne, H. Z. Bootsma, R. C. S. Mason, N. Skalko, G. Gregoriadis, and J. H. L. Playfair.** 1992. Antibodies against phosphatidylinositol and inositol monophosphate specifically inhibit TNF production by malaria exoantigens. Immunology **76:**31–35.
4. **Bate, C. A. W., J. Taverne, E. Roman, C. Moreno, and J. H. L. Playfair.** 1991. TNF induction by malaria exoantigens depends on phospholipid. Immunology **75:**129–135.
5. **Berendt, A. R., D. L. Simmons, J. Tansey, C. I. Newbold, and K. Marsh.** 1989. Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum*. Nature (London) **341:**57–59.
6. **Carlson, J., H. Helmby, A. V. S. Hill, D. Brewster, B. M. Greenwood, and M. Wahlgren.** 1990. Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies. Lancet **336:**1457–1460.
7. **Clark, I. A.** 1987. Cell-mediated immunity in protection and pathology of malaria. Parasitol. Today **3:**300–305.
8. **Clark, I. A., K. A. Rockett, and W. B. Cowden.** 1992. Proposed central role of nitric oxide in conditions clinically similar to cerebral malaria. Lancet **340:**894–895.
9. **Grau, G. E., L. F. Fajardo, P.-F. Piguet, B. Allet, P.-H. Lambert, and P. Vassali.** 1987. Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. Science **237:**1210–1212.
10. **Grau, G. E., T. E. Taylor, M. E. Molyneux, J. J. Wirima, P. Vassalli, M. Hommel, and P.-H. Lambert.** Tumor necrosis factor and disease severity in children with falciparum malaria. N. Engl. J. Med. **320:**1586–1591.
11. **Hill, A. V. S., C. E. M. Allsopp, D. Kwiatkowski, N. M. Anstey, P. Twumasi, P. A. Rowe, S. Bennett, D. Brewster, A. J. McMichael, and B. M. Greenwood.** 1991. Common West African HLA antigens are associated with protection from severe malaria. Nature (London) **352:**595–600.
12. **Ho, M., T. M. Davis, K. Silamut, D. Bunnag, and N. J. White.** 1991. Rosette formation of *Plasmodium falciparum*-infected erythrocytes from patients with acute malaria. Infect. Immun. **59:**2135–2139.
13. **Kwiatkowski, D.** TNF-inducing toxins and the regulation of malaria parasitaemia. Parasitol. Today, in press.
14. **Kwiatkowski, D., J. G. Cannon, K. R. Manogue, A. Cerami, C. A. Dinarello, and B. M. Greenwood.** 1989. Tumour necrosis factor production in falciparum malaria and its association with schizont rupture. Clin. Exp. Immunol. **77:**361–366.
15. **Kwiatkowski, D., A. V. S. Hill, I. Sambou, P. Twumasi, J. Castracane, K. R. Manogue, A. Cerami, D. R. Brewster, and B. M. Greenwood.** 1990. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. Lancet **336:**1201–1204.
16. **MacPherson, G. G., M. J. Warrell, N. J. White, S. Looareesuwan, and D. A. Warrell.** 1985. Human cerebral malaria: a quantitative ultrastructural analysis of parasitized erythrocyte sequestration. Am. J. Pathol. **119:**385–401.
17. **McGuire, W., A. V. S. Hill, C. E. M. Allsopp, B. M. Greenwood, and D. Kwiatkowski.** 1994. Variation in the TNF-alpha promoter region is associated with susceptibility to cerebral malaria. Nature (London) **371:**508–511.
18. **Schofield, L., and F. Hackett.** 1993. Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites. J. Exp. Med. **177:** 145–153.