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Tumour necrosis factor production in *Falciparum* malaria and its association with schizont rupture

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SUMMARY

To investigate the involvement of tumour necrosis factor (TNF) in human malaria, we studied TNF production in patients infected with *Plasmodium falciparum*, and in co-cultures of human mononuclear cells and malaria parasites *in vitro*. In the examined sample, plasma TNF levels of over 39 pg/ml were detected in the plasma of 59% of Gambian children with acute malaria, 17% of convalescents, 9% of children with mild infections other than malaria, and 7% of healthy Gambian adults. Mononuclear cells of acute malaria patients, when stimulated with endotoxin *in vitro*, secreted twice as much TNF as did those of convalescent individuals, and three times that of healthy adult controls. Erythrocytic cultures of *P. falciparum* stimulated increased TNF secretion by mononuclear cells from uninfected individuals, and a sharp rise in the rate of secretion occurred shortly after schizont rupture. We suggest that malaria fever is mediated, at least in part, through paroxysmal TNF release associated with schizont rupture.

Keywords *Plasmodium falciparum* tumour necrosis factor fever

INTRODUCTION

Tumour necrosis factor (TNF), also known as cachectin, is a polypeptide secreted by monocytes and macrophages, which induces various metabolic, inflammatory, and immunological changes associated with infection (Beutler & Cerami, 1987). It is an endogenous pyrogen (Dinarello *et al.*, 1986) and a major mediator of endotoxic shock (Tracey *et al.*, 1987).

Experimental studies in mice have demonstrated that significant amounts of TNF are released in murine malaria (Clark, 1978; Hotez *et al.*, 1984; Taverne, Treagust & Playfair, 1986; Bate, Taverne & Playfair, 1988). There is evidence that TNF can mediate host defence against the malaria parasite (Taverne *et al.*, 1987; Clark *et al.*, 1987) but that it can also cause severe pathology, including cerebral complications, in infected mice (Clark, 1978; Grau *et al.*, 1987; Clark, 1987). There are fewer data concerning TNF and human malaria, but elevated TNF levels in acute malaria sera have been reported (Scuderi *et al.*, 1986; van der Meer *et al.*, 1988) and a number of studies concerning its clinical significance are in progress.

In this paper we describe plasma TNF levels in Gambian children with *falciparum* malaria, in the acute illness and in convalescence. Because isolated circulating TNF levels could conceivably be unrepresentative of its rate of production, we have also investigated *in vitro* TNF secretion by mononuclear cells of patients with malaria. Finally, to determine whether TNF may be released in response to a particular event in the parasite life cycle, we have studied co-cultures of parasitized erythrocytes and human mononuclear cells.

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SUBJECTS

Subjects

We studied 75 Gambian children who presented to clinic at Fajara between July and December 1987 with acute symptoms of malaria due to *Plasmodium falciparum*. Mean age was 5·5 years (s.d. 3·1), mean duration of illness 2·9 days (s.d. 1·9), mean parasitaemia 104 000/μl (s.d. 100 000) and mean temperature 39·0°C (s.d. 1·2). Nineteen of the patients had severe malaria, defined as unrousable coma or circulatory collapse; they were admitted and treated with parenteral chloroquine. Three patients died within 24 h of admission. The remaining 56 patients received oral chloroquine, mostly as outpatients. Children with haemoglobin < 6 g/dl were excluded from this study for ethical reasons. Venous blood was collected before treatment and clinical status was recorded, including temperature, weight, level of consciousness, blood glucose (in the severe group), parasitaemia, haemoglobin, and white blood cell count. A convalescent blood sample was taken at 3 to 4 weeks or at 6 to 12 weeks after treatment, when the patient was asymptomatic, afebrile, and free of parasitaemia.

Controls

Controls included 55 healthy Gambian adults (mean age 30·2 years, s.d. 8·4); five Europeans resident in The Gambia (mean age 31·0 years, s.d. 3·8); and 22 Gambian children (mean age 4·5 years, s.d. 3·9) who presented to the clinic with mild infections other than malaria. The latter group had a mean temperature of 37·9°C (s.d. 1·1), and comprised 14 patients with respiratory infections, five patients with gastoenteritis, and three others with minor complaints. None of the controls were parasitaemic at the time of sampling.

Determination of TNF in plasma

Blood was collected onto EDTA (1 mg/ml) and aprotinin (0·5 TIU/ml), and platelet-free plasma was stored at −20°C. TNF levels were determined by ELISA as previously described (Michie *et al.*, 1988) using recombinant human TNF (rhTNF) and purified monoclonal antibody to rhTNF (SDW 18.1.1) kindly provided by Chiron Corp. (Emeryville, CA). The assay detected TNF at concentrations of $\geqslant 39$ pg/ml. Since plasma TNF levels were distributed around the detection limit of the assay, results were analysed using non-parametric statistics.

TNF production by mononuclear cells from patients and controls

Heparinized blood was obtained from 19 acute malaria patients, 14 convalescent individuals (3 weeks post-treatment), and 11 healthy adults (six Gambian and five European). Using pyrogen-free materials, mononuclear cells were isolated on Lymphoprep (Nyegaard, Oslo, Norway), washed, and dispensed into flat-bottomed microtitre wells (5×10^5 cells/well) in 200 μl MEM + HEPES, penicillin, streptomycin and 1% **AB**+ human serum, with or without 50 ng/ml lipopolysaccharide (*Escherichia coli* 055:B5, Sigma Chemical Co., St Louis, MO). The proportion of monocytes and lymphocytes was estimated by counting Giemsa-stained slides. After 16-h incubation in a 5% CO_2 atmosphere at 37°C, supernatants were harvested and stored at −20°C. TNF levels were measured by competitive radioimmunoassay, as previously described (van der Meer *et al.*, 1988).

Stimulation of TNF production by P. falciparum in vitro

Peripheral blood mononuclear cells were obtained, as above, from healthy uninfected adults (three Gambian, three European) and 10^7 cells were dispensed into 35-mm culture dishes with 2·5 ml of endotoxin-free RPMI + HEPES, gentamicin, and 10% non-immune human serum. Cryopreserved erythrocytes infected with *P. falciparum* (obtained from an adult Gambian patient) were thawed, washed four times, and 100 μl packed cells were added to the mononuclear cell cultures, which were then incubated in candle jars at 37°C. The initial parasitaemia was 3–4%, and at least 95% of parasites were at the young ring stage of development. Control cultures contained mononuclear cells plus unparasitised erythrocytes from a healthy Gambian adult. The rate of TNF secretion was determined by aspirating the culture medium and replacing with fresh medium every 6 h. Aspirated medium was centrifuged to remove cells and stored with aprotinin (0·5 TIU/ml) at −20°C prior to TNF assay. Parasite development was assessed on Giemsa-stained thin films.

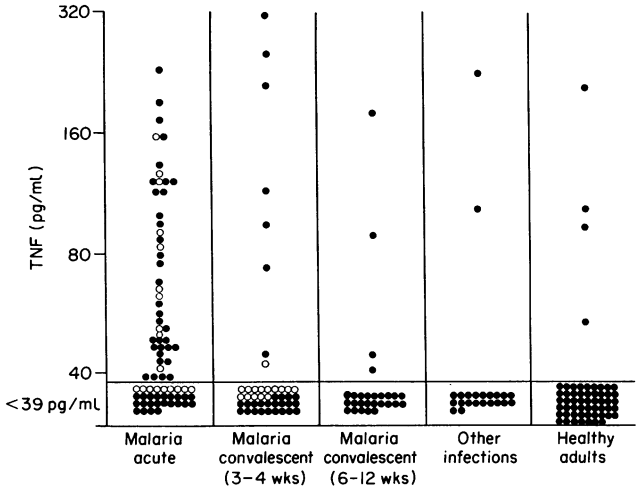

Fig. 1. Plasma TNF levels in patients with malaria, children with mild infections other than malaria, and healthy adult controls. Open circles represent patients with severe malaria.

RESULTS

Plasma TNF levels

Data are shown in Fig. 1. High TNF levels were seen more frequently in acute malaria patients than in convalescents ($P < 0.0001$, Wilcoxon matched pairs), healthy adults ($P < 0.0001$, Mann–Whitney *U*-test) or children with mild infections other than malaria ($P < 0.001$, Mann–Whitney). TNF was detected in 44 out of 75 (59%) acute malaria patients, in 12 out of 72 (17%) convalescents, in four out of 56 (7%) healthy adults, and in two out of 22 (9%) children with non-malaria infection. The range of TNF levels observed was similar in all groups.

Circulating TNF was detected in 34 out of 56 (61%) of children with non-severe malaria, 10 out of 19 (53%) of those with severe malaria, and in only one of the three children who died (156 pg/ml). Scatter plots and linear regression analysis failed to reveal any relation between pre-treatment TNF levels and measurements of temperature, parasitaemia, haemoglobin, neutrophil count, or (in the severe group) blood glucose (data not shown).

Sequential blood samples were taken from eight patients with severe malaria. Five had detectable TNF levels prior to treatment; 24 h after treatment was commenced only one patient had detectable TNF, and this was less than the initial level.

Among non-severe malaria patients, seven out of 29 (24%) had detectable TNF at 3–4 weeks after treatment, and four out of 27 (15%) at 6–12 weeks: levels in the 3–4 week follow-up group differed significantly from those of healthy adults ($P < 0.02$, Mann–Whitney *U*-test). In the severe group, only one of the 16 survivors had detectable TNF at follow up. No correlation was observed between acute and convalescent levels in individual patients.

TNF production in vitro: *malaria patients and controls*

When stimulated with endotoxin (Fig. 2) mononuclear cells of acute malaria patients secreted significantly more TNF than did those of convalescents ($P < 0.02$ by two-tailed Student's *t*-test) or healthy adults ($P < 0.005$). A similar tendency was seen in the

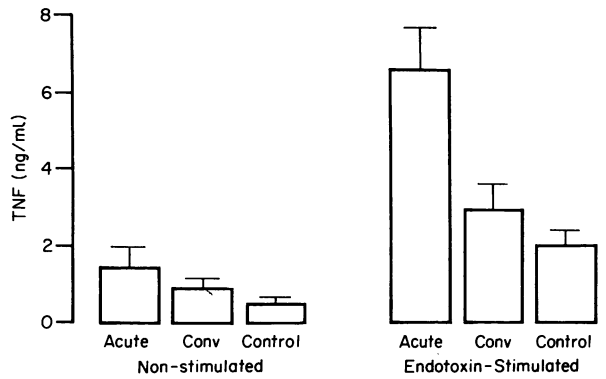

Fig. 2. Mean TNF production by mononuclear cells of acute malaria patients ($n=19$), convalescent individuals ($n=14$) and healthy adult controls ($n=11$), with and without endotoxin stimulation. Bars represent s.e.m.

Table 1. Peripheral blood monocyte density and endotoxin-stimulated TNF production *in vitro*

	Malaria patients		Healthy controls ($n=11$)
	Acute ($n=19$)	Convalescent ($n=14$)	
Absolute monocyte density in peripheral blood ($\times 10^6/l$)	630±113	286±42	241±22
TNF yield of total mononuclear cell fraction (pg/10^3 cells)	2·63±0·42	1·17±0·26	0·79±0·16
Corrected TNF yield (pg/10^3 monocytes)	17·8±2·4	16·0±3·0	9·1±1·7

Results are presented as mean±s.e.m. For correction of TNF yield see Results.

absence of endotoxin stimulation, but was not statistically significant.

More circulating monocytes were present in acute malaria patients than in convalescent individuals ($P<0·02$, two-tailed t-test) or in healthy adults ($P<0·001$), as shown in Table 1. Since altered monocyte numbers could significantly influence TNF production, the TNF yield of the total mononuclear cell fraction was divided by the estimated number of cultured monocytes. This value represents a maximum estimate of the TNF yield per monocyte—since lymphocytes may have also contributed to the total output (Cuturi *et al.*, 1987)—and was higher for acute malaria patients than healthy adults ($P<0·02$, two-tailed t-test). Convalescent malaria patients also had a higher value than did healthy adults, but this difference was not statistically significant ($0·1>P>0·05$).

TNF production by mononuclear cells stimulated with P. falciparum *in vitro*

Malaria parasites cultured for 72 h in the presence of mononuclear cells from uninfected adults developed normally from

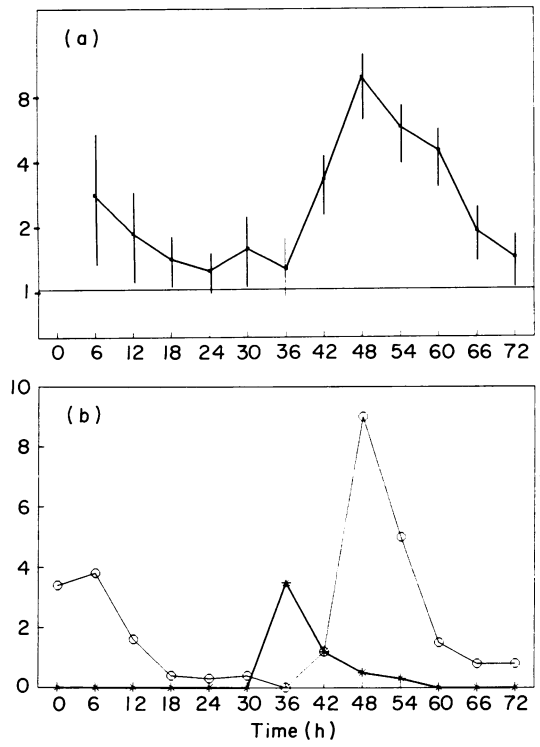

Fig. 3. (a) TNF production in co-cultures of mononuclear cells and parasitized erythrocytes, expressed as a stimulation index (see Results). Geometric mean with standard error; (b) Time course of parasite development in a representative co-culture. ○, rings; *, schizonts.

the ring stage to schizont rupture, and a second generation of ring-stage parasites appeared at 44–48 h (Fig. 3, lower panel). The supernatant was sampled periodically, as described above, and the rate of TNF secretion was estimated for each sampling period (Fig. 4). In the control cultures (mononuclear cells + unparasitized erythrocytes) the rate of TNF secretion declined gradually over the first 24 h, but was otherwise reasonably stable throughout the experiment. Two individuals (one Gambian, one European) had markedly higher rates of secretion than the other four subjects. In the experimental cultures (mononuclear cells + parasitized erythrocytes) the pattern was significantly different. Over the first 24 h, rates of TNF secretion for four individuals were similar to those in the control cultures, whereas the other two had markedly elevated rates of secretion at the start of the experiment: these were the two who had the higher control values. At 30 and 36 h, levels were similar in experimental and control cultures for all subjects. At 42–48 h, as the number of schizonts fell and young rings appeared in the parasite cultures (i.e. following schizont rupture), all six experimental cultures exhibited an increase in the rate of TNF secretion and then subsided towards basal levels over the course of the next 18 h. The observed rate of TNF secretion was significantly higher in experimental than in control cultures at 42, 48, 54 and 60 h (in each case, $P<0·02$ by two-tailed paired t-test on logarithmically transformed data) but not at any of the other time points recorded. A TNF stimulation index was defined as the rate of secretion in an experimental culture at a given time point, divided by the rate of secretion in the

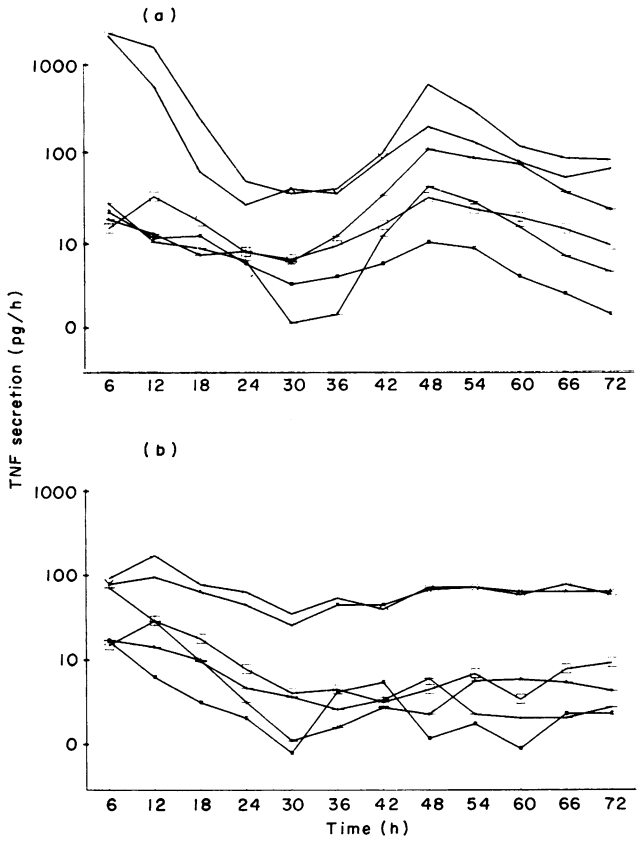

Fig. 4. TNF secretion by mononuclear cells cultured in the presence of parasitized (a) or unparasitized (b) erythrocytes. Mononuclear cell donors were three uninfected Gambian adults (◇, □, *) and three Europeans (×, +, ■).

corresponding control culture (Fig. 3, upper panel). The geometric mean stimulation index at 48 h (8·9, 95% confidence interval 4·5–17·5) was significantly higher than at any other time after the first 12 h of the experiment, with the exception of 54 h ($P < 0{\cdot}02$ for each comparison by paired t-test on logarithmically transformed data). Inspection of Fig. 4 reveals no consistent difference between the European and Gambian donors.

DISCUSSION

This study provides three forms of evidence that TNF production is increased in malaria due to *P. falciparum*. Firstly, high plasma TNF levels are detected significantly more often in Gambian children with acute malaria than in convalescent individuals, children with mild infections other than malaria, or healthy Gambian adults. Secondly, mononuclear cells from patients with malaria secrete considerably more TNF in response to endotoxin *in vitro* than do cells from healthy individuals. Thirdly, erythrocytic cultures of *P. falciparum* stimulate TNF secretion by mononuclear cells *in vitro*, and there is a temporal relation between the life cycle of the parasite and TNF release.

Interpretation of the plasma TNF data is complex. For instance, there was no apparent correlation between TNF levels and disease severity in the 75 patients that we describe: how can this be reconciled with the hypothesis that TNF is an important pathological mediator in this disease? It does not negate the hypothesis, since there are several possible confounding factors. Firstly, it is conceivable that our ELISA can detect both biologically active and inactive forms of the molecule (Petersen & Moller, 1988). Secondly, individual patients may differ in their sensitivity to a given amount of TNF, and it seems likely that this can be modulated by inhibitors (Seckinger, Isaaz & Dayer, 1988) or synergistic cytokines (Waage *et al.*, 1989). Thirdly, our *in vitro* findings (discussed below) indicate that TNF release in malaria is likely to be intermittent. This means that isolated acute phase TNF levels should be interpreted with caution, since TNF is rapidly cleared from plasma by receptor binding (Beutler, Milsark & Cerami, 1985) and its clinical effects may persist for some time after circulating TNF has fallen to undetectable levels (Michie *et al.*, 1988; Hesse *et al.*, 1988). There is evidence that a transient massive spike in circulating TNF levels (20 000 pg/ml) can mediate fatal bacteraemic shock in baboons, with undetectable TNF levels (< 35 pg/ml) in the terminal phase of the illness (Tracey *et al.*, 1987; Hesse *et al.*, 1988). In this study, patients had been unwell for an average of 3 days before presenting to clinic, and most of the 19 patients with severe malaria had to travel far to get to the clinic. These considerations may conceivably explain the lack of correlation between TNF levels and clinical status in the patients reported here, and a more extensive study focussing on severe malaria is in progress.

Further investigation is also needed to explain our observation that convalescent patients had high plasma TNF levels more frequently than did controls (our *in vitro* data are consistent with this) despite being asymptomatic at the time. It raises the possibility that recent malaria infection may cause an individual to become refractory to the clinical effects of TNF, and this may also partly explain why the parasitaemia necessary to cause fever rises with repeated infection (Kitchen, 1949) and how *vivax* malaria can induce endotoxin tolerance (Rubinstein *et al.*, 1965).

Since the level of circulating TNF depends on its rate of clearance as well as the rate of production, and because clearance by receptor binding may be altered in disease (Aggarwal, Eesalu & Hass, 1985), we sought to confirm that TNF production is increased in malaria by studying *in vitro* TNF secretion by peripheral blood mononuclear cells of malaria patients and controls. Although monocytes are the major producers of TNF in the peripheral blood, we used the undepleted mononuclear population (monocytes plus lymphocytes) because lymphocytes produce cytokines that modulate TNF secretion by monocytes (Nedwin *et al.*, 1985) and are also capable of TNF production (Cuturi *et al.*, 1987). In response to endotoxin, the total mononuclear cell fraction from children with malaria produced on average twice as much TNF as cells of convalescent children, and three times that of healthy adult controls. Adult controls were used for ethical reasons, because of the exploratory nature of this part of the study and the additional amount of blood required, and further investigations using age-matched controls are indicated.

Acute malaria patients had more circulating monocytes than did convalescent patients or healthy adults, and this factor may have confounded the above data. However, after correction for the estimated number of monocytes in culture, we found that endotoxin-stimulated TNF production remained significantly elevated in acute malaria patients compared with healthy controls. The value for convalescents was also higher than controls, but this difference was not statistically significant. A further potential confounding factor is that recent massive stimulation of mononuclear cells *in vivo* could possibly make them transiently refractory for TNF production: if this is so, then the real difference between acute malaria patients and controls may be greater than we have observed.

We therefore propose that at least two factors may enhance the TNF-productive capacity of peripheral blood in malaria: an acute influx of circulating monocytes; and a priming of monocytes (and possibly also lymphocytes) for TNF production, which may persist for some weeks after the acute episode.

TNF is a potent endogenous pyrogen (Dinarello *et al.*, 1986). Since fever, the principal clinical manifestation of malaria, is related to the rupture of erythrocytic schizonts and the appearance of rings in the circulation (Golgi, 1889) we sought an association between the life cycle of the parasite and TNF release. However, this relation is difficult to investigate in clinical malaria for several reasons: *P. falciparum* schizonts are not seen in peripheral blood, infection is not always synchronous, and sequential studies in untreated patients are not ethically feasible. We report preliminary data from an *in vitro* model which demonstrate that *P. falciparum*, in its asexual erythrocytic phase of growth, stimulates intermittent TNF secretion by human mononuclear cells (Fig. 3). To compensate for variability in the TNF responsiveness of cultured mononuclear cells during the 72 h of observation, we have expressed our final data as a stimulation index that compares TNF production in experimental cultures (containing parasitized erythrocytes) and in control cultures (containing unparasitized erythrocytes). Two peaks were seen. The smaller peak, at the start of the experiment, was not statistically significant and may be an artefact of our initial manipulations. The larger peak was statistically significant: it occurred at 48 h, shortly after schizont rupture. (Another small peak was observed at 96 h in cultures that were continued through a second growth cycle; data not shown). Thus TNF secretion *in vitro* and fever *in vivo* obey the same temporal relation to the parasite life cycle, providing circumstantial evidence that TNF is a mediator of malaria fever. The precise stimulus of TNF release requires further investigation.

It has been proposed that TNF may contribute to the pathology of severe malaria (Clark, 1978; 1987). From our data we conclude that TNF release in malaria, like fever, is paroxysmal; and that massive release may occur when a critical number of schizonts rupture simultaneously in the presence of mononuclear cells already primed for TNF production.

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