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Levels of tumour necrosis factor and soluble TNF receptors during malaria fever episodes in the community

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Abstract

The pyrogenic cytokine, tumour necrosis factor (TNF), is a mediator of malaria fever. Since high plasma levels of TNF are sometimes found in afebrile individuals with *Plasmodium falciparum* parasitaemia, it has been suggested that soluble forms of TNF receptors (sTNF-R55 and sTNF-R75) in the plasma may act to inhibit the pyrogenic effect of TNF. We have investigated plasma levels of TNF, sTNF-R55 and sTNF-R75 in relation to episodes of malaria fever detected in a cross-sectional study of 313 rural Gambian children during the malaria transmission season. Levels of TNF were significantly higher in the 20 children who had parasitaemia associated with fever than in 120 children who were afebrile despite malaria infection and 173 who had no detectable parasitaemia. In contrast, soluble TNF receptor levels did not differ between these clinical groups and, in a logistic regression model which included level of parasitaemia, we found TNF but not soluble TNF receptor levels to be associated with the presence of fever. These data support the role of TNF in malaria fever but suggest that soluble TNF receptors are not a major factor in modulating the fever.

Keywords: malaria, *Plasmodium falciparum*, fever, pathogenesis, tumour necrosis factor, children, The Gambia

Introduction

The immunological processes that lead to clinical tolerance of malaria infection are poorly understood. Fever, the predominant symptom of malaria, is caused by factors released at schizont rupture (GOLGI, 1889) that stimulate the host to produce tumour necrosis factor (TNF) and other pyrogenic cytokines (KWIATKOWSKI *et al.*, 1989, 1993). However, elevated levels of TNF have been detected in the plasma of children with *Plasmodium falciparum* infection when they are afebrile (PEYRON *et al.*, 1990; MSHANA *et al.*, 1991). Similarly it has been proposed that TNF is a major factor in the pathogenesis of cerebral malaria. Several studies have demonstrated an association between high plasma TNF and malaria disease severity, yet there is considerable overlap in the TNF levels between those with mild and those with severe disease (CLARK, 1987; GRAU *et al.*, 1989; KWIATKOWSKI *et al.*, 1990). These observations raise the question of whether clinical tolerance might arise, at least in part, from inhibitory factors that modulate the biological activity of TNF in the circulation of the infected individual.

One such mechanism could be the production of inhibitory TNF-binding proteins. The 2 cell-surface receptors for TNF also exist as soluble forms, termed sTNF-R55 and sTNF-R75, and are present in the plasma of malaria patients (KERN *et al.*, 1992; MOLYNEUX *et al.*, 1993). There is evidence of inhibition of the biological activity of TNF by sTNF-R55 and sTNF-R75 *in vitro* (SECKINGER *et al.*, 1988; ENGELMANN *et al.*, 1990), raising the question of whether they act to modulate the clinical effects of TNF bioactivity in the context of natural infection. This study considered the possibility that soluble TNF receptors might act to modulate the pyrogenic effect of TNF in malaria, thus explaining why some parasitaemic individuals seem to tolerate high circulating levels of TNF while remaining afebrile. We measured plasma TNF, sTNF-R55 and sTNF-R75 in a cross-sectional sample of rural Gambian children during the malaria transmission season, to determine their respective associations with malaria fever episodes in a natural endemic setting.

Subjects and Methods

During the malaria transmission season (October–November 1992), a cross-sectional clinical survey of a random sample of 313 rural Gambian children, aged 1–4 years, was undertaken (see D'ALESSANDRO *et al.*, 1995 for details). The epidemiology of malaria in rural Gambia has been described previously (THOMSON *et al.*, 1994). The children were examined, their axillary temperature measured and a finger-prick blood sample collected into tubes containing sodium ethylenediaminetetraacetate (1 mg/mL) and aprotinin (0·5 trypsin inhibitor units/mL). Plasma was separated immediately by centrifugation at 10000 ***g*** and stored frozen until assayed. Thick blood films were stained with Field's stain and parasitaemic individuals were treated appropriately. A second thick blood film was stained with Giemsa's stain and 100 microscope fields were examined (×1000); parasite density was estimated assuming that one parasite per field is equivalent to 500/μL (GREENWOOD & ARMSTRONG, 1991).

Measurement of TNF and sTNF-Rs

TNF levels were measured by an immunoradiometric assay (IRMA; Medgenix, Belgium) as described previously (KWIATKOWSKI *et al.*, 1993). This detection system measures total plasma TNF, including that already bound to soluble receptors (ENGELBERTS *et al.*, 1991). The lower limit of detection of the IRMA was 15pg/mL, and for statistical purposes values less than this were entered as 10pg/mL.

Plasma sTNF-R55 and sTNF-R75 were assayed by separate enzyme linked immunosorbant assays (GARDINER *et al.*, 1995). Briefly, microtitre plates were coated with the relevant capture monoclonal antibody (murine anti-human sTNF-R55 or -R75). Samples and serial dilutions of standards were then added. After incubation and washing, a second biotin-conjugated murine monoclonal antibody against human sTNF-R55 or sTNF-R75 was applied. After further incubation and washing a streptavidin–horse radish peroxidase conjugate was added, incubated, then washed before tetramethylbenzidine substrate was added. The optical density at 630nm (reference 420nm) of the developed product was measured and levels of receptor estimated by interpolation on a standard curve. Under these conditions, the lower limit of detection was 0·5ng/mL for sTNF-R55 and 5ng/mL for sTNF-R75. For statistical purposes values below these were attributed concentrations of 0·2 ng/mL and 2ng/mL respectively. The antibodies were the gift of Celltech Limited, UK.

Assays were performed on samples subjected to no more than 2 freeze/thaw cycles.

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Statistical analysis

Means were compared between groups by Mann–Whitney *U* tests and correlations by Spearman's rank correlation.

Ethical clearance

All aspects of the study were approved by the Gambia Government/MRC Joint Ethical Committee. Prior consent was obtained from the guardians of all children included.

Results

In all, 313 children, 51% female, were studied. The median age was 2·4 years. The most common ethnic group was Mandinka (33%), followed by Fula (23%), Sarahuli (19%), Jola (17%), and Wollof (6%).

Clinical profile

Three clinical categories were defined. The first category included 20 children (6·4% of the study population) who had detectable asexual parasitaemia associated with an axillary temperature of 37·5°C or greater and no other obvious cause of fever (P+F+). The second category included 120 children (38·3%) who were afebrile despite infection with *P. falciparum* (P+F–). The third category comprised 173 children (55·3%) who had no detectable parasitaemia (P–). Three of the aparasitaemic individuals had axillary temperatures greater than 37·5°C. There was no significant difference in age, sex or ethnic group distribution between these groups.

The geometric mean parasite count was significantly greater in the P+F+ group (14660/μL, 95% confidence interval [95% CI] 5901–36421) than in the P+F– group (610/μL, 95% CI 366–1016; $P<0·001$).

Relationship of TNF and sTNF-Rs to parasitaemia and fever

There was a significant positive association between level of parasitaemia and levels of TNF ($r_s = 0·32$, $P<0·001$), sTNF-R55 ($r_s = 0·24$, $P<0·01$) and sTNF-R75 ($r_s = 0·26$, $P<0·01$) (Fig.1).

Geometric mean levels of TNF were higher in the P+F+ group (51 pg/mL) than in the P+F– group (21 pg/mL; $P<0·001$), and higher in the P+F– group than in the P– group (21 pg/mL versus 15 pg/mL; $P = 0·003$). In contrast, neither sTNF-R55 nor sTNF-R75 levels differed significantly among the 3 clinical categories (Fig. 2).

To examine whether soluble TNF receptor levels affected the association between TNF and fever in parasitaemic children, we used a logistic regression analysis. When the analysis was restricted to TNF, sTNF-R55 and sTNF-R75, the only significant predictor of fever was TNF, suggesting that soluble TNF receptor levels do not significantly affect the ability of TNF to induce fever in this clinical context (Wald coefficient 8·8, $P=0·003$; all variables were logarithmically transformed). TNF remained a significant predictor of fever after correcting for level of parasitaemia by including this in the logistic regression model (Wald statistic for parasitaemia 9·2, $P=0·002$; Wald statistic for TNF 4·0, $P=0·045$; all variables were logarithmically transformed).

Discussion

In this community-based study of rural Gambian children we found elevated circulating TNF levels to be associated with episodes of malaria fever. This association held true after correction for level of parasitaemia in a logistic regression model, and it was not affected by plasma levels of the soluble TNF receptors, sTNF-R55 and sTNF-R75. These data complement a growing body of evidence that TNF mediates malaria fever, but do not support the hypothesis that inhibitory TNF-binding proteins are an important mechanism of clinical tolerance in African children with malaria.

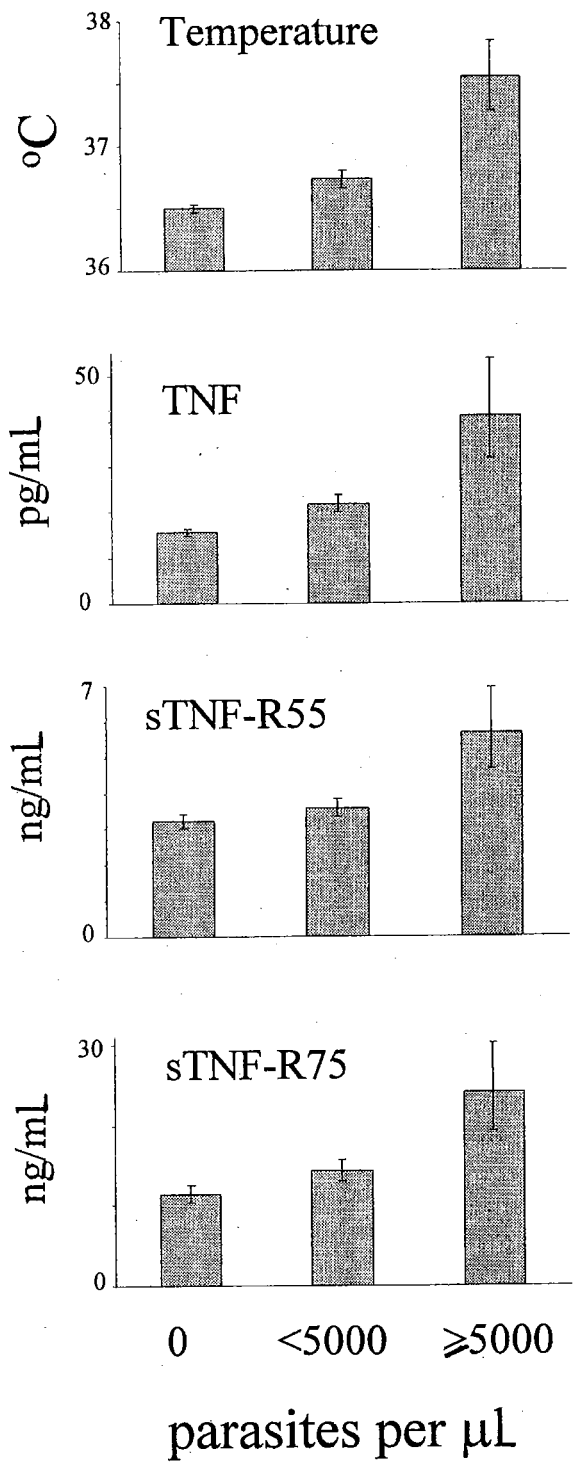

Fig. 1. Mean temperature and geometric mean levels of tumour necrosis factor (TNF) and the soluble TNF receptors sTNF-R55 and sTNF-R75, plotted in relation to parasite density in 313 Gambian children. 173 children had no parasites, 122 had <5000 parasites per μL, and 18 had ≥5000 parasites per μL. Error bars represent 95% confidence intervals.

Several lines of evidence support the role of TNF as a critical mediator of malaria fever: it is a potent endogenous pyrogen (DINARELLO *et al.*, 1986) which is released by human peripheral blood mononuclear cells in response to schizont rupture (KWIATKOWSKI *et al.*, 1989), and monoclonal anti-TNF antibodies suppress fever in children with cerebral malaria (KWIATKOWSKI

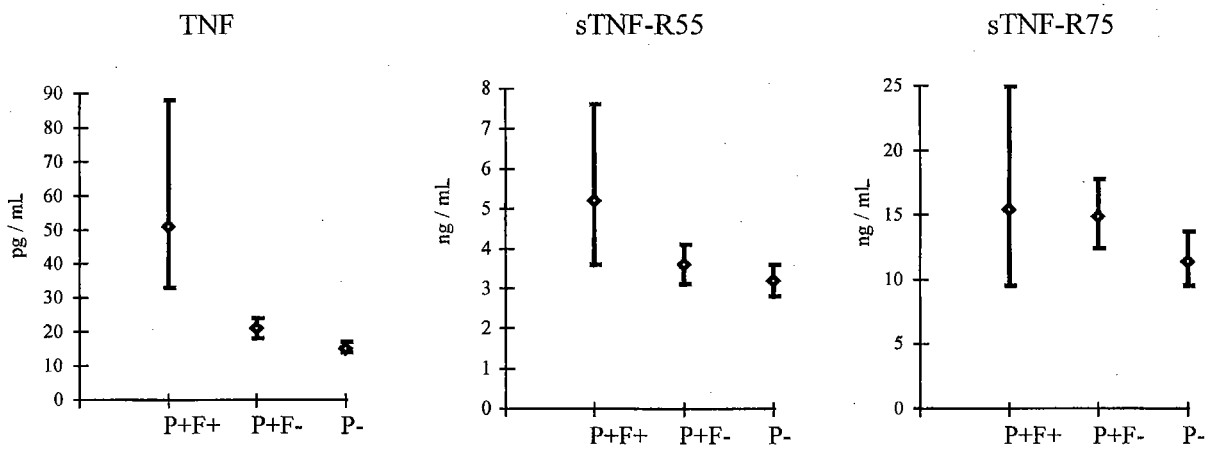

Fig. 2. Geometric mean levels of tumour necrosis factor (TNF) and the soluble TNF receptors sTNF-R55 and sTNF-R75, plotted in relation to clinical category in 313 Gambian children. 173 children had no parasitaemia (P–), 120 had parasitaemia but were afebrile (P+F–), and 20 had parasitaemia with fever (P+F+). Error bars represent 95% confidence intervals.

et al., 1993; BOELE VAN HENSBROEK *et al.*, 1996). In *P. vivax* malaria, a temporal relationship has been demonstrated between elevated plasma TNF and fever paroxysms, by sequential clinical measurements before the commencement of antimalarial treatment (KARUNAWEERA *et al.*, 1992). Such temporal correlations are more difficult to investigate in *P. falciparum* infection, partly because fever paroxysms are less clearly defined, but also because it is not acceptable to delay antimalarial treatment once a diagnosis of symptomatic falciparum malaria has been made. We therefore examined a random cross-section of rural Gambian children during the malaria transmission season, expecting that a significant proportion would be found to be parasitaemic and that some of these would be at a febrile stage of the infection. We investigated children aged 1 to 4 years as this group bears the brunt of malaria morbidity in The Gambia (GREENWOOD *et al.*, 1987). Information obtained from this cross-sectional sample should represent events at the various stages in the natural history of *P. falciparum* infection as it is likely that a proportion of the afebrile parasitaemic children would have been febrile in previous days or would have become febrile subsequently (TRAPE *et al.*, 1985).

It is likely that a variety of infectious agents contributes to increased TNF production in this population, but these data indicate that malaria is a predominant cause since plasma TNF levels were significantly correlated with parasite density at the community level. We found that plasma TNF levels were significantly higher in children who were both parasitaemic and febrile at the time of examination, compared to those who were parasitaemic but afebrile. Although parasitaemia was significantly lower in the latter group, logistic regression analysis indicated that at least part of the association between TNF and fever was independent of the level of parasitaemia.

The TNF assay method employed in this study gives an indication of total circulating TNF levels and has been shown to be relatively unaffected by the presence of soluble TNF receptors (ENGELBERTS *et al.*, 1991). We detected sTNF-R55 and sTNF-R75 levels in the ng/mL range, which represents a 100–1000-fold molar excess over TNF level in the plasma, yet the data provided no evidence that these inhibitory binding proteins act to modulate the pyrogenic effect of TNF in malaria infected individuals. That is, amongst children who were parasitaemic, soluble TNF receptor levels did not differ significantly between those who were febrile and those who were not. Furthermore, inclusion of sTNF-R55 and sTNF-R75 levels in a logistic regression model did not weaken the association of TNF level with fever. Thus we are left with our original question of why some children remain asymptomatic despite elevated plasma TNF levels. A possible technical explanation might be that artefactual elevation of TNF can result from the presence of rheumatoid factors or other components that cross-react with TNF immunassays; while a biological explanation might be that malaria fever depends on synergy between a number of different pyrogenic cytokines, of which TNF is simply the best-studied example.

The function of soluble TNF receptors in the context of malaria remains unclear. Even in healthy individuals, sTNF-Rs are found in plasma at ng/mL concentrations (ADERKA *et al.*, 1992a). They are derived by proteolytic cleavage from the cell surface forms (NOPHAR *et al.*, 1990). TNF itself is thought to contribute to TNF receptor cleavage (LANTZ *et al.*, 1990), and our observed correlation of TNF with sTNF-R55 and sTNF-R75 levels would be consistent with this mechanism. It may be that the soluble receptors act to buffer the endocrine effects of circulating TNF (SPINAS *et al.*, 1992), but there is also experimental evidence to suggest that in some circumstances they might act to prolong the bioactive half-life of TNF within the circulation (ADERKA *et al.*, 1992b). Although this is the first study to investigate the possible effects of soluble TNF receptors on malaria fever symptoms, it has been previously documented that high levels are found in patients with severe complications of *P. falciparum* infection (KERN *et al.*, 1992; MOLYNEUX *et al.*, 1993; KREMSNER *et al.*, 1995). In the latter 2 studies, in Malawian and Gabonese children respectively, it was noted that the ratio of TNF to soluble TNF receptors was higher in severe than in uncomplicated malaria, and on this basis it was suggested that soluble TNF receptors may act to attenuate the serious adverse effects of TNF in malaria. However, it is difficult in falciparum malaria to obtain the detailed sequential data that might allow investigation of the dynamic interplay between TNF and its soluble receptors before the onset of severe symptoms. The physiological function of circulating soluble TNF receptors in malaria and other infectious diseases remains a matter of considerable clinical interest.

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