The host inflammatory response in the pathogenesis of malaria in Gambian children

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A thesis submitted for the
Degree of MD
Faculty of Medicine
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October 1998

Abstract

The host inflammatory response may be involved in the pathogenesis of severe malaria. High plasma levels of pro-inflammatory cytokines, notably tumour necrosis factor (TNF), are found in African children with cerebral malaria. Experimental evidence also implicates TNF in the pathogenic process of severe malarial anaemia. We have examined the association of polymorphisms in the TNF gene promoter region with severe malaria in a large case-control study of Gambian children. Cerebral malaria, but not severe malarial anaemia, was found to be associated with the TNF_{-308} A promoter allele (TNF2). Homozygotes for this allele had a relative risk of 7 for death or severe neurological sequelae due to cerebral malaria and this association was independent of variation in the neighbouring HLA class I and class II alleles. In the same population severe malarial anaemia was associated with the TNF_{-238} A promoter allele with an odds ratio of 2.5 after stratification for HLA type. These data suggest that regulatory polymorphisms of cytokine genes can affect the outcome of severe infection and that severe malarial anaemia and cerebral malaria are influenced by separate genetic factors situated in the neighbourhood of the TNF gene.

TNF is an endogenous pyrogen and a critical mediator of malaria fever. However, high plasma levels of TNF are sometimes found in afebrile African children with *Plasmodium falciparum* parasitaemia. A proposed mechanism for this apparent clinical tolerance is that soluble forms of TNF receptors (sTNF-R55 and sTNF-R75), that are known to inhibit TNF bioactivity *in vitro*, modulate the pyrogenic effect of TNF *in vivo*. We have measured the plasma levels of TNF, sTNF-R55 and sTNF-R75 in relation to episodes of malaria fever detected in a cross-sectional study of rural Gambian children during the malaria transmission season. TNF levels were significantly higher in children who were both parasitaemic and febrile, compared to those who were parasitaemic but afebrile and those who had no detectable parasitaemia. In contrast, soluble TNF receptor levels did not differ between these clinical groups and, in a logistic regression model that included level of parasitaemia, TNF but not soluble TNF receptor levels was 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.

Sequestration of parasitised erythrocytes in post-capillary venules in critical areas of the brain is believed to be a critical step in the pathogenesis of cerebral malaria. High levels of pro-inflammatory cytokine production may exacerbate sequestration by up-regulating the expression of the specific endothelial receptors to which parasitised erythrocytes bind. One of the best characterised of these receptors is intercellular adhesion molecule-1 (ICAM-1). An indirect measure of ICAM-1 expression may be provided by the amount of circulating ICAM-1 (cICAM-1) in the plasma. In a case-control study of severe malaria, we have found that the plasma levels of cICAM-1 to be higher in Gambian children with acute malaria than in those with non-malarial illnesses. cICAM-1 levels correlated with levels of TNF, interleukin-1 alpha (IL-1) and interferon-gamma, supporting the view that these cytokines are responsible for a general up-regulation of ICAM-1 expression in malaria. However, in contrast to the finding for TNF and IL-1, cICAM-1 levels were unrelated to malaria disease severity. The tissue distribution of ICAM-1 expression, rather than the total level of expression may be the more important determinant of whether an individual child develops cerebral malaria.

The host acute inflammatory response to malaria parasitaemia may provide a useful surrogate measure of disease activity. Two such potential measures of malarial morbidity are (i) elevated plasma C-reactive protein (CRP) plus detectable parasitaemia, as an indicator of the malaria-induced acute-phase response; and (ii) reduced plasma haptoglobin, which in children resident in malaria endemic areas of sub-Saharan Africa indicates malaria-induced intravascular haemolysis. We have compared these indices with the traditional malariometric indices in a cross-sectional survey that was undertaken to determine the impact of bed net usage in 1505 rural Gambian children. At the end of the malaria transmission season the proportion of children who had parasitaemia plus elevated CRP was significantly lower than in those who had slept under insecticide-treated bed nets compared to those who did not use a bed net. The proportion with a low haptoglobin differed similarly. We conclude that these indices could be useful for the assessment of the impact of intervention programmes on malarial morbidity in rural African communities.

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I

General Introduction

Plasmodium falciparum malaria remains endemic in many areas of sub-Saharan Africa. Young children bear the brunt of morbidity and mortality; older children and adults have acquired clinical immunity. The best estimates suggest that around 200 million clinical episodes of falciparum malaria occur each year in African children under the age of five years. The majority of these are episodes of **mild malaria**; parasitaemia resulting in a self-limiting febrile illness. Life-threatening disease develops in only a minority of instances (approximately 1-2%), but causes up to 2 million childhood deaths in Africa annually (Greenwood *et al.*, 1991). An improved understanding of the pathogenesis of malaria and of the nature of clinical immunity is essential if better methods of reducing the huge burden of malaria morbidity and mortality are to be developed (Marsh *et al.*, 1992).

Severe malaria in African children

In children living in malaria endemic areas of sub-Saharan Africa the most important severe complications of *P. falciparum* infection are **cerebral malaria** (coma, often in association with seizures) and **severe malarial anaemia** (usually defined as haemoglobin <5g/dl associated with asexual *P. falciparum* parasitaemia) (Warrell *et al.*, 1990). Although these conditions can be considered as clinically distinct, occurring at peak incidence in different age groups, children can present with both conditions simultaneously (Brewster *et al* , 1990). More recently the importance of additional indicators of severe disease, such as respiratory distress associated with acidosis, hypoglycaemia, and poor peripheral perfusion, has been highlighted (Marsh *et al.*, 1995; English *et al.*, 1996, 1997). Hepatic failure, renal failure and pulmonary oedema, features of severe malaria that are common in non-immune adults, are rarely seen in African children (Warrell *et al.*, 1990).

The mortality from malaria in African children is high despite effective anti-malarial therapy. A hospital-based study of Gambian children with cerebral malaria documented a fatality rate of 16%, with a further 9% affected by major neurological sequelae such as hemiplegia and cortical blindness (Brewster *et al.*, 1990). Given the low level of health care provision throughout much of rural Africa, it is likely that many children die before reaching hospital. In The Gambia the overall malaria mortality rate in children under the age of 5 years is estimated at 6.1 per 1000 (Greenwood & Pickering, 1993). The widespread emergence of chloroquine-resistant *P. falciparum* may increase this rate still further (Marsh, 1998).

Prospects for malaria control

Global malaria eradication is now regarded as unfeasible and the current World Health Organisation emphasis has shifted to malaria control. Community-based measures that reduce mosquito contact, such as insecticide-impregnated bed net and curtain use, have been shown to reduce malarial morbidity and mortality, but the sustainability of this form of intervention is unclear (D'Alessandro *et al.*, 1995a; Binka *et al.*, 1996; Nevill *et al.*, 1996). Chemoprophylaxis may be of some value in particular circumstances, such as pregnancy, but is unlikely to be a useful long-term preventative strategy for the vast majority of susceptible children (Greenwood, 1997). With regard to vaccine development, the only malaria vaccine so far tested in large field studies has been the synthetic polypeptide vaccine SPf66. Although a trial in Tanzania did indicate that SPf66 provided some protection against clinical malaria (Alonso *et al.*, 1994), a large prospective study in Gambian infants failed to show any significant degree of protection (D'Alessandro *et al.*, 1995b). Field trails of two other malaria vaccines are currently underway, but no major vaccine candidates is likely to be in extensive use in near future. Insecticide-impregnated bed nets probably offer the best option for malaria control at present and renewed efforts should be directed towards improving the sustainability of this intervention (D'Alessandro, 1997; Greenwood, 1997).

Why do only a minority of children develop severe disease?

African children resident in malaria endemic areas are repeatedly infected with *Plasmodium falciparum*, but in only a minority of instances does infection progress to severe disease (Fig. 1). It is likely that many interacting factors, including vector and parasite factors, socio-economic, behavioural and other host factors, contribute to the overall risk of disease progression (Table 1). An improved understanding of how these factors determine the clinical consequence of *P. falciparum* infection may help in the development of better or novel approaches for treatment or control (Greenwood *et al.*, 1991; Marsh *et al.*, 1992).

Fig. 1. The possible outcome of inoculation with malaria sporozoites. The numbers indicate how many African children are likely to fall into each category for every child who dies from malaria. The proportion of infected children progressing to symptomatic disease will vary, being low in areas of very high endemicity. Modified with permission from Greenwood *et al.*, 1991.

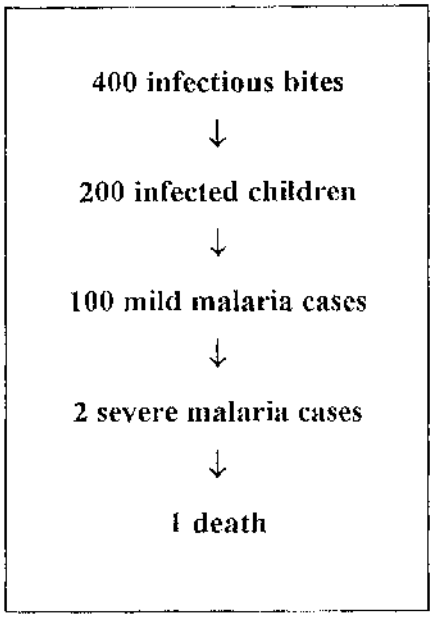

Table 1. Factors which may influence the clinical outcome of malaria infection.

Vector:	Multiple factors affecting cumulative sporozoite dose.
Parasite:	Inter-dependent factors; growth, cytoadherence, immune evasion and drug resistance.
Host:	Level of acquired immunity, nutritional status, genetic susceptibility. Availability of health care, community usage of anti-malarials, local mosquito avoidance practices.

Host genetic factors affecting disease susceptibility

The role of host genetic factors in affecting susceptibility to severe malaria was first illustrated by epidemiological findings of a high prevalence of red blood cell abnormalities, such as sickle cell trait, thalassaemia and glucose-6-phosphate dehydrogenase (G6PD) deficiency, in areas of high malaria endemicity (Allison, 1954, 1960; Willcox *et al.*, 1983; Flint *et al.*, 1986). More recently the degree of protection conferred by these polymorphisms has been quantified (Hill *et al.*, 1991a; Ruwende *et al.*, 1995; Yates *et al.*, 1995). Intriguingly, a recent study in Vanuatu has indicated that alpha-thalassaemia may protect against severe falciparum malaria by increasing susceptibility to *P.vivax* infection in young childhood and affording a degree of cross-species protection (Williams *et al.*, 1996). This situation does not exist in the West Africa population, where absence of the Duffy blood group antigen on the red cell surface prevents invasion by *P.*vivax (Miller *et al,* 1976).

The role of immune response genes, particularly human leucocyte antigen (HLA) class I and class II alleles, in determining the clinical consequence of infection has also been investigated. A large case-control study of severe malaria in Gambian children demonstrated an association of the HLA class I allele, HLA-B53, with protection against cerebral malaria and severe malarial anaemia, and of the HLA class II allele, HLA-DRB1*1302, with protection against severe malarial anaemia (Hill *et al.*, 1991a).

The host inflammatory response in the pathogenesis of severe malaria

In addition to HLA-class I and class II alleles, many other host genes are involved in the immune response to infection and the role played by the host cytokine response in the pathogenesis of malaria has been a subject of considerable interest. Attention has focused on the pro-inflammatory cytokines interleukin-1 (IL-1), IL-6 and, in particular, tumour necrosis factor (TNF). TNF is released by host monocytic cells in response to products of ruptured malaria schizonts (Kwiatkowski, 1989). TNF stimulates various processes that can inhibit the growth of *P.falciparum*, including fever, phagocytosis, and the release of nitric oxide and reactive oxygen intermediates (Clark *et al.*, 1987; Kwiatkowski, 1989; Taylor-Robinson *et al.*, 1993). Several studies have demonstrated an association between elevated plasma concentrations of TNF and malaria disease severity. Therefore, although it is

recognised that TNF and other pro-inflammatory cytokines are critical mediators of host defence, it has been proposed that excessive generation of TNF may contribute to the development of severe disease, particularly cerebral malaria (Grau *et al.*, 1989; Kern *et al.*, 1989; Kwiatkowski *et al.*, 1990).

TNF may be involved in the development of cerebral malaria by up-regulating the expression of endothelial adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 and E-selectin, which mediate the cytoadherence of infected erythrocytes to cerebral blood vessels (Berendt *et al.*, 1989; Ockenhouse *et al.*, 1992a). TNF may contribute to the hypoglycaemia and lactic acidosis commonly associated with cerebral malaria (Krishna *et al.*, 1994). It has also been proposed that TNF production at sites of parasitised erythrocyte sequestration may induce the release of nitric oxide by vascular endothelium, thus perturbing neurotransmission (Clark *et al.*, 1992).

With regard to severe malarial anaemia, the pathogenic process is multifactorial and only partly explained by direct destruction of erythrocytes (Weatherall, 1993). Studies in Gambian children have indicated that the suppression of erythropoiesis may be of importance (Abdalla *et al.*, 1980). Several lines of experimental evidence suggest that erythropoiesis can be severely disrupted by TNF. (Clark & Chuadhri, 1988; Johnson *et al.*, 1989; Taverne *et al.*, 1994). It may be that chronic low-grade production of TNF contributes to the pathogenesis of malarial anaemia but there are little clinical data available to support this theory.

The series of papers presented in this thesis relate to the further investigation of various aspects of the role of TNF and the host acute inflammatory response in the pathogenesis of falciparum malaria in African children. Four related areas of work are described, over the following five chapters, as summarised below:

TNF promoter polymorphisms and severe malaria **(Chapters II and III)**
We have applied a genetic approach to the investigation of the role of TNF in severe malaria in Gambian children. The gene for TNF resides within the class III region of the major

histocompatability complex, approximately 250kb centromeric of HLA-B and 900kb telomeric of HLA-DR. The first polymorphism to be described in the TNF promoter region, at -308 nucleotides relative to the transcription start site, was identified in a Northern European population by Wilson *et al.* (1992). A second polymorphism, at -238 nucleotides in the TNF promoter, was identified in an Italian population (D'Alfonso & Richiardi, 1994). As described above, in a large case-control study in Gambian children undertaken between 1988 and 1990, Hill *et al.* (1991a) have demonstrated the association of severe malaria with HLA class I and class II alleles. Using this library of clinical samples we have now examined the association between clinical condition and TNF promoter polymorphisms.

Inhibitory TNF binding proteins in malaria fever (**Chapter IV**)

TNF is an endogenous pyrogen, but high plasma levels have been found in parasitaemic children who are afebrile (Peyron *et al.*, 1990; Mshana *et al.*, 1991). We wished to explore the possibility that circulating forms of the two cell-surface receptors for TNF (sTNF-R1 and sTNF-R2), which have been demonstrated to inhibit the bioactivity of TNF *in vitro* (Seckinger *et al.*, 1988; Engelmann *et al.*, 1990), modulate the pyrogenic activity of TNF *in vivo*.We undertook a survey of rural Gambian children during the malaria transmission season and measured the plasma levels of TNF, sTNF-R1 and sTNF-R2 in order to determine their respective associations with malaria fever episodes.

Up-regulation of ICAM-1 in cerebral malaria (**Chapter V**)

An important factor in the pathogenesis of cerebral malaria may be TNF-mediated up-regulation of endothelial receptors for parasitised erythrocytes as this may lead to increased sequestration at critical sites in the brain. This is consistent with the pathogenic process seen in one murine model of malaria, where TNF-induced over-expression of ICAM-1, a well-characterised endothelial receptor for parasitised erythrocytes (Berendt *et al.*, 1989, 1992), underlies the observed neuro-pathological changes (Grau *et al.*, 1987, 1991). The plasma level of the shed form of ICAM-1 (sICAM-1) is a potentially useful surrogate indicator of tissue ICAM-1 expression. We measured sICAM-1 in children recruited to the Gambian case-control study of severe malaria in order to determine if cerebral malaria was associated with increased generalised expression of ICAM-1 compared to children with uncomplicated malarial illness.

The inflammatory response as a marker of disease in the community **(Chapter VI)**

As discussed previously, in African children living in malaria endemic regions, malarial morbidity is not indicated solely by the presence of parasitaemia (Marsh, 1992). An awareness of the role of the host inflammatory response in the malaria disease process has led to the suggestion that stable biochemical markers of the acute-phase response may be developed as surrogate indicators of disease activity for use in community surveys (Rougemont *et al.*, 1988; Sisay *et al.*, 1992; Hurt *et al.*, 1994). The aim of this study was to investigate the use of the acute phase proteins C-reactive protein (CRP), which is generated in response to pro-inflammatory cytokines, and haptoglobin, which indicates intra-vascular haemolysis, as surrogate markers of malaria morbidity. To accomplish this, we surveyed 1505 Gambian children at the end of the malaria transmission season and assessed the impact a community- based malaria intervention programme (insecticide-impregnated bed net usage) on conventional malariometric indices and on the plasma levels of CRP and haptoglobin.

II

Variation in the TNF promoter region associated with susceptibility to cerebral malaria

[1]McGuire, W., [2]Hill, A.V.S., [2]Allsopp, C.E.M., [3]Greenwood, B.M. & [1]Kwiatkowski, D. (1994). *Nature*, **371**, 508-511.

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Summary

TNF is believed to have an important role in the pathogenesis of severe infectious disease and fatal cerebral malaria is associated with high circulating levels of this cytokine. In a large case-control study of malaria in Gambian children we have found that homozygotes for the TNF_{-308} A allele, a variant of the TNF gene promoter region, have a relative risk of 7 for death or severe neurological sequelae due to cerebral malaria. Although the TNF_{-308} A allele is in linkage disequilibrium with several neighbouring HLA alleles, we have shown that this disease association is independent of HLA class I and class II variation. These data suggest that regulatory polymorphisms of cytokine genes can affect the outcome of severe infection. The maintenance of the TNF_{-308} A allele at a gene frequency of 0.16 in The Gambia implies that the increased risk of cerebral malaria in homozygotes is counterbalanced by some biological advantage.

Introduction

It is not known why cerebral malaria occurs in only a small proportion of individuals infected with *Plasmodium falciparum*. A critical factor may be the amount of TNF that is produced when malaria schizonts rupture (Kwiatkowski, 1989). Clinical studies have demonstrated an association between TNF levels and disease severity (Grau *et al.*, 1989; Kern *et al.*, 1989; Kwiatkowski *et al.*, 1990) and experimental studies have revealed several ways in which excessive TNF production could promote cerebral malaria (Clark *et al.*, 1987; Grau *et al.*, 1987). A prominent feature of this condition is parasite sequestration within cerebral blood vessels, and TNF is known to up-regulate the expression of host adhesion molecules that mediate the binding of parasites to vascular endothelium (Berendt *et al.*, 1989). It has also been proposed that local high concentrations of TNF may stimulate cerebral endothelium to overproduce nitric oxide, thus causing coma by interfering with neurotransmission (Clark *et al.*, 1992).

These observations raise the possibility that cerebral malaria is related to the genetic propensity of the host to produce TNF. The gene for TNF resides within the class III region of the major histocompatibility complex (MHC) and several studies have shown that

individual differences in TNF production can be linked to HLA type and other polymorphic markers in this region (Molvig *et al.*, 1988; Jacob *et al.*, 1990; Pociot *et al.*, 1993). This suggests that TNF responsiveness is controlled by variable genetic elements within the MHC but the precise location of these elements has yet to be identified. A polymorphism that may directly affect the regulation of TNF is located at -308 nucleotides relative to the transcriptional start site of the gene (Wilson *et al.*, 1992, 1993). There are two allelic forms, referred to as TNF_{-308} G (originally termed TNF1) and TNF_{-308} A (originally termed TNF2) that are identical apart from a single base transition (G to A) at position -308 of TNF. Studies of the TNF promoter linked to a chloramphenicol acetyl transferase reporter gene have shown that the TNF_{-308} A allele is associated with higher constitutive and inducible levels of transcription than the TNF_{-308} G allele (Wilson *et al.*, 1994). Because of the possible functional significance of this polymorphism, we have investigated its association with cerebral malaria.

Subjects and Methods

Our analysis was based on a large case-control study of severe malaria in Gambian children. The details of this study have been described previously (Hill *et al.*, 1991a). Children aged 1 to 10 years were enrolled, with the permission of a parent or guardian, at the Royal Victoria Hospital, Banjul, the MRC Clinic, Fajara, and neighbouring health centres during the 1988-1990 rainy seasons. Cerebral malaria was defined as a Blantyre coma score <3 (persisting for >30 minutes after effective treatment of hypoglycaemia or convulsions) or repeated prolonged seizures (>30 minutes) in a child with *P. falciparum* parasitaemia and no other apparent cause of fits or coma. A further group of children with severe malarial anaemia but without cerebral complications was also investigated. Severe malarial anaemia was defined as haemoglobin <5g/dl in association with asexual *P. falciparum* parasitaemia of ≥2500/ul.

The severe malaria group was matched to the two control groups for area of residence around Banjul. Our primary control group was composed of children who presented to clinic with a variety of mild illnesses other than malaria; all were free of parasitaemia and well enough to be treated as outpatients. These 'mild non-malaria' controls were used to estimate background allele frequencies and relative risks. Around 65% had upper

respiratory tract infections, mild chest infections, or otitis media; 25% had mild gastroenteritis or other abdominal complaints and 10% had other minor illnesses. The second group were children with an uncomplicated febrile illness with asexual *Plasmodium falciparum* parasitaemia and no other evident cause of fever. These 'mild malaria' patients were used to test the hypothesis that the TNF_{-308} A allele predisposes to cerebral malaria rather than malaria illness in general.

Typing of the TNF gene

DNA was extracted by standard methods from venous blood samples. To type the polymorphism, polymerase chain reaction (PCR) was used to amplify a 519 base pair fragment of the TNF promoter region, spanning -502 to +17 nucleotides relative to the transcriptional start site as defined by Nedospasov *et al.* (1986). Approximately 100ng of genomic DNA extracted from venous blood was added to 25ul reaction mixture containing 0.1uM of each primer (5'-CAAACACAGGCCTCAGGACTC-3' and 5'-AGGGAGCGTCTGCTGGCTG-3') with 100uM of each dNTP (Boehringer Mannheim, Germany), 67mM Tris-HCl, 16mM $(NH_4)_2SO_4$, 2mM $MgCl_2$, and 0.01% Tween-20. After heating at 95°C for 10 minutes, 1.5U of Taq DNA polymerase (Bioline, UK) was added, followed by 35 cycles at 95°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, then a final 10 minutes at 72°C. Template-free controls were included in each experiment. The PCR product was denatured with 0.4M NaOH, dotted onto nylon membrane via a vacuum manifold, and fixed for 1 minute in UV light. Allele-specific oligonucleotides (5'-AGGGGCATGGGGACGGG-3' for the TNF_{-308} G allele and 5'-AGGGGCATGAGGACGGG-3' for the TNF_{-308} A allele) were end-labeled with digoxigenin-ddUTP (Boehringer Mannheim, Germany) and hybridised to the membranes in 3M tetramethylammonium chloride (TMAC) solution at 55°C for 1 hour. Excess probe was removed by washing in 2x SSPE/0.1% SDS at room temperature and subsequently in 3M TMAC at 58°C. The membranes were treated with anti-digoxigenin Fab fragments and, after washing to remove excess antibody, they were incubated with Lumigen PPD ddUTP (Boehringer Mannheim, Germany) and exposed to X-ray film for 15-30 minutes. Two independent observers scored allele type. The accuracy of this method was confirmed by DNA sequencing of the PCR product, as described below, in three TNF_{-308} G homozygotes, three TNF_{-308} A homozygotes, and four heterozygotes.

To explore the possibility that the TNF_{-308} A allele might be linked to some other regulatory polymorphism, we sequenced between -502 and +250 nucleotides relative to the transcriptional start site of the TNF gene in 20 individuals who were typed by PCR as TNF2 homozygotes. The PCR product described above was purified and directly sequenced using the cycle sequencing technique (US Biochemical, USA) with the internal oligonucleotide 5'-TCCTGCATCCTGTCTGGAAG-3' in a labeling reaction which omitted dCTP. In addition, we used the primers 5'-CCGCTGGTTGAATGATTCT-3' and 5'-TGATCCTGAAGAGGAGAGA-3' to amplify a second overlapping PCR product which was analysed over the region -73 to +250 using the internal oligonucleotide 5'-CCTCCTCTCGCCCCAGGG-3' (omitting dGTP in the labelling reaction).

HLA typing

Class I types were analysed by serological testing of fresh or cryopreserved lymphocytes, using 180 well-characterised antisera in a standard microlymphocytotoxicity assay. The apparent homozygote frequencies given in Table 5 tend to be overestimates of the true homozygote rate because of the presence of serologically undetected alleles, particularly the values for HLA-C. HLA-B53 antigen types were either determined serologically or by allele-group-specific PCR amplification and oligonucleotide hybridisation as described by Allsopp *et al.* (1991). Class II types were determined by restriction fragment length polymorphisms using *Taq*I digestion with DRB- and DQB- specific probes, supplemented by PCR-oligonucleotide analysis as described by Hill *et al.* (1992). The full range of HLA types identified is reported by Hill *et al.* (1991b).

Statistical analysis

Clinical groups were compared by X^2 test, using stratified Mantel-Haenszel weighted analysis to correct for the possible confounding effect of ethnic heterogeneity and to examine for interactions with neighbouring HLA alleles.

Ethical approval

The study was approved by the Gambian Government / Medical Research Council Joint Ethical Committee.

Results

Three hundred and seventy-six cases of cerebral malaria were compared with two groups of controls recruited at the same clinics, frequency matched for area of residence (Table 2). The cerebral malaria sample included 45 children who also had severe malarial anaemia. 72 of the children with cerebral malaria died. A further 27 had disabling neurological sequelae when discharged from the ward. The typical clinical features have been previously described (Brewster *et al.*, 1990). Deaths and severe neurological sequelae were combined in this analysis because of the previous finding that both of these adverse outcomes tend to be associated with high TNF levels (Kwiatkowski *et al.*, 1990).

The mean age of the cerebral malaria cases was 3.9 years; mild malaria controls, 3.9 years; mild non-malaria controls, 2.9 years; severe malarial anaemia cases, 2.3 years. The ethnic composition of the study population was Mandinka (45%), Jola (15%), Wolof (14%), Fula (11%), and other groups (15%).

Association of the TNF_{-308} A allele with presenting clinical condition

The gene frequency of the TNF_{-308} A allele in this population was 0.16, and its distribution in the control groups was consistent with Hardy-Weinberg equilibrium. Among cases of cerebral malaria there was a significantly higher frequency of TNF_{-308} A homozygotes than in non-malaria controls (X^2=6.5, P=0.01) or mild malaria controls (X^2=4.1, P=0.04). The frequency of TNF_{-308} A homozygotes was even higher in those cases of cerebral malaria who died or developed gross neurological sequelae (Table 2). This was analysed by Fisher exact test, because of the small number of homozygotes involved, and found to be significantly different from non-malaria controls (P=0.001) or mild malaria controls (P=0.005). Cases of severe malarial anaemia without cerebral complications had a similar frequency of TNF_{-308} A homozygotes to the two control groups. A detailed breakdown of homozygote frequencies by ethnic group is given in Table 3. Using the mild non-malaria controls to represent the background population and stratifying for ethnic group, we estimate that TNF_{-308} A homozygotes have a relative risk of 4.0 for cerebral malaria (95% confidence interval (CI), 1.2-13.8; X^2 =5.4, P=0.02, Mantel-Haenszel test) and of 7.7 for death or sequelae due to cerebral malaria (95% CI, 1.9-30.0; X^2=10.2, P=0.001).

Table 2. TNF_{-308} A allele and homozygote frequencies by clinical category.

	Sample size	TNF_{-308} A homozygotes %	TNF_{-308} A heterozygotes %
Mild non-malaria	325	1.2	29.8
Mild malaria	332	1.8	26.8
Severe malaria anaemia	111	1.8	31.5
Cerebral malaria			
All cases	376	4.5	26.6
Deaths and sequelae	99	8.1	25.3

Table 3. TNF_{-308} A homozygote frequencies stratified by ethnic group (sample size in parentheses).

	Mandinka	Jola	Wolof	Fula	Other
Mild non-malaria controls	2.0% (151)	0.0% (41)	0.0% (38)	0.0% (35)	1.7% (60)
Cerebral malaria					
All cases	3.9% (153)	8.1% (62)	5.1% (59)	2.3% (44)	3.4% (58)
Deaths and sequelae	7.0% (43)	15.0% (20)	0.0% (13)	9.1% (11)	8.3% (12)

Significant associations between the TNF_{-308} A allele and HLA phenotypes that are present at ≥2% frequency in this population are shown in Table 4. Because of the multiple comparisons made, *P* values were corrected by multiplying the number of concurrent tests performed, namely 31 class I types and 18 class II types. TNF_{-308} A was not associated with any HLA-A type. HLA-A1, B8 and DR3 were specifically analysed because of their close linkage to TNF_{-308} A in Europeans (Wilson *et al.*, 1993) but they were not associated with TNF_{-308} A in this population.

Table 4. HLA phenotypes associated with the TNF_{-308} A allele (P_{corr}: *P* value corrected for multiple comparisons).

	TNF_{-308} A +	TNF_{-308} A - (i.e., TNF_{-308} G homozygotes)	Odds Ratio
B70+	43	39	2.7, P_{corr}=0.0009
B70-	140	345	
B50+	19	8	5.5, P_{corr}=0.0005
B50-	164	376	
Cw2+	44	39	2.8, P_{corr}=0.0005
Cw2-	139	345	
DRB1*13.02+	148	234	1.7, P_{corr}=0.0005
DRB1*13.02-	295	788	
DRB1*11.01+	117	112	2.9, P_{corr}=1.3 x 10^{-12}
DRB1*11.01-	326	910	
DRB1*13.03+	8	56	0.3, P_{corr}=0.03
DRB1*13.03-	435	966	

The frequencies of the TNF_{-308} A allele -linked HLA class I and class II phenotypes in mild non-malaria controls and cases of cerebral malaria are shown in Tables 5 and 6. Homoygote and heterozygote frequencies were analysed separately because we specifically wanted to exclude the possibility that the increased disease susceptibility of TNF_{-308} A homozygotes could be attributed to an HLA association. The HLA alleles that were positively associated with TNF_{-308} A were not associated with increased susceptibility to cerebral malaria, and the negatively-associated HLA allele was not associated with protection.

Table 5. Frequency of HLA class I types associated with TNF_{-308} A in mild non-malaria controls and cases of cerebral malaria.

	Controls (n=144)		Cerebral malaria (n=215)	
	Apparent homozygotes	Heterozygotes	Apparent homozygotes	Heterozygotes
B70	3.5	11.8	0.5	14.0
B50	0.0	2.8	0.5	5.1
Cw2	(4.2)	7.6	(6.0)	11.2

Table 6. Frequency of HLA class II types associated with TNF_{-308} A in mild non-malaria controls and cases of cerebral malaria.

	Controls (n=325)		Cerebral malaria (n=374)	
	Homozygotes	Heterozygotes	Homozygotes	Heterozygotes
DRB1*13.02	4.6	24.3	1.3	24.1
DRB1*11.01	0.0	14.8	0.0	15.5
DRB1*13.03	0.0	3.4	0.0	5.1

Analysis of possible interactions between TNF_{-308} A and HLA-B53

HLA-B53 is associated with protection from severe malaria in this population. Typing of 636 Gambian children without malaria showed that the frequency of the TNF_{-308} A phenotype was slightly lower in HLA-B53 positive than HLA-B53 negative individuals, but this was not statistically significant (42/148 *versus* 152/488; odds ratio=0.88, P=0.52), that is, there was no evidence of a strong negative association between TNF_{-308} A and HLA-B53. The data in Table 7 show the relationship between TNF_{-308} A homozygote frequency and disease severity, after stratifying for HLA-B53 phenotype. When these stratified data were analysed by Mantel-Haenszel test, TNF_{-308} A homozygotes had a relative risk (compared to mild non-malaria controls) of 4.1 for cerebral malaria (X^2 =5.7, P=0.02) and 7.0 for death or sequelae due to cerebral malaria (X^2=9.3, P=0.002), showing that the TNF_{-308} A association is independent of HLA-B53.

Table 7. TNF_{-308} A homozygote frequencies by clinical category, stratified by HLA-B53 phenotype.

	HLA-B53 positive		HLA-B53 negative	
	n	TNF_{-308} A homozygotes %	*n*	TNF_{-308} A homozygotes %
Mild non-malaria	91	2.2	233	0.9
Mild malaria	80	0.0	251	2.4
Cerebral malaria				
All cases	59	6.8	308	3.9
Deaths and sequelae	20	10.0	73	6.8

Discussion

Several features suggest that this polymorphism in the TNF gene may be a causal predisposing factor for cerebral malaria. The available evidence from transfection studies indicates that the TNF_{-308} A allele acts to increase the transcription of TNF (Wilson *et al.*, 1994). This is consistent with our observation that homozygotes for the TNF_{-308} A allele are at increased risk for cerebral malaria, as we have previously found that Gambian children with cerebral malaria have higher TNF levels than children with mild malaria (Kwiatkowski *et al.*, 1990). TNF levels are highest in those children who die or develop neurological sequelae due to cerebral malaria and it is in this group that we found the highest frequency of TNF_{-308} A homozygotes. The lack of an association with severe malaria anaemia is consistent with the view that acute excess of TNF production is specifically involved in the pathogenesis of cerebral malaria, rather than severe malaria in general (Kwiatkowski, 1992).

We recognise that the TNF_{-308} A allele could be associated with some other allele affecting TNF regulation, although only one other polymorphism in the TNF gene has so far been described, at position -238 nucleotides relative to the transcriptional start site (D'Alfonso & Richiardi, 1994). We sequenced the region of the TNF gene that is most likely to be involved in transcriptional regulation (Goldfeld *et al.*, 1990, 1991; Rhoades *et al.*, 1992) in 20 individuals who were typed by PCR as TNF_{-308} A homozygotes. This sequence included much of the promoter as well as the 5' untranslated region. No polymorphisms were found other than the single base substitution at position –308.

As the TNF gene is located at approximately 250 kb centromeric of HLA-B and 1Mb telomeric of HLA-DR (Dunham *et al.*, 1987), we considered the possibility that this association could be due to linkage disequilibrium with neighbouring HLA class I or class II antigens. We found that HLA-B70, B50, Cw2, DRB1*13.02 and DRB1*11.01 were associated with the TNF_{-308} A allele in our study population, yet none of these HLA types was associated with susceptibility to cerebral malaria . Conversely, DRB1*13.03 was negatively associated with the TNF_{-308} A allele but was not associated with protection.

We specifically analysed the relationship of TNF_{-308} A with HLA-B53, which is associated with resistance to severe malaria in Gambian children (Hill *et al.*, 1991). In 636 children without malaria we found only a weak negative association between these two alleles (which was not statistically significant), and a Mantel-Haenszel test confirmed that the association of TNF_{-308} A with cerebral malaria is independent of HLA-B53 status. Several lines of evidence indicate that these two neighbouring alleles affect malaria outcome through entirely different mechanisms. The protective effect of HLA-B53 seems to be equally strong for homozygotes and heterozygotes, whereas the TNF_{-308} A association is confined to homozygotes. HLA-B53 is protective against both cerebral malaria and severe malaria anaemia, whereas the TNF_{-308} A allele is only associated with cerebral malaria. Excessive TNF production is thought to have a specific role in the pathogenesis of cerebral malaria (Clark *et al.*, 1987; Grau *et al.*, 1987; Berendt *et al.*, 1989; Clark *et al.*, 1992), while HLA-B53 probably acts by promoting an effective cytotoxic T-cell response against the parasite in the pre-erythrocytic stage of infection (Hill *et al.*, 1992).

Malaria has been a powerful agent for the selection of genetic polymorphisms which protect the host against severe complications of the infection. The TNF_{-308} A allele exists at a gene frequency of 0.16 in The Gambia despite its association with cerebral malaria, implying that the disadvantage for TNF_{-308} A homozygotes is counter-balanced by some biological advantage. This may well be in relation to host-defence, as TNF promotes a variety of anti-microbial mechanisms in malaria and other infections (Kwiatkowski, 1989; Titus *et al.*, 1991; Grau *et al.*, 1992; Wong *et al.*, 1992). It is possible that the TNF_{-308} A allele is maintained because heterozygotes possess an optimal level of TNF response against a broad range of infections; alternatively it is possible that TNF_{-308} A homozygotes are themselves protected against life-threatening conditions other than cerebral malaria.

III

Severe malarial anaemia and cerebral malaria are associated with different TNF promoter alleles

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Summary

Experimental evidence implicates TNF in the pathogenesis of malarial anaemia, but there are few data relating to this hypothesis. We report that severely anaemic children with *Plasmodium falciparum* infection have low plasma TNF levels, in contrast to the high levels found in cerebral malaria. A previous case-control study in The Gambia found cerebral malaria, but not severe malarial anaemia, to be associated with the TNF_{-308} A allele. Here we report that in the same population severe malarial anaemia is associated with the TNF_{-238} A allele with an odds ratio of 2.5 (P=0.0006) after stratification for HLA type. These findings suggest that severe malarial anaemia and cerebral malaria are influenced by separate genetic factors situated in the neighbourhood of the TNF gene.

Introduction

Around half a million African children die each year as a consequence of severe malarial anaemia. However, although most children in sub-Saharan Africa are repeatedly infected with *Plasmodium falciparum*, life-threatening anaemia develops in only a minority. It is unclear which factors predispose these children to become markedly more anaemic than others (Greenwood *et al.*, 1991).

The pathogenic process is of malarial anaemia is multifactorial and is only partly explained by direct destruction of erythrocytes by parasites (Weatherall, 1993). Previous studies in The Gambia have indicated that suppression of erythropoiesis may be of additional importance. Many children with severe anaemia were found to have a depressed reticulocyte response, and a significant proportion of children had gross morphological abnormalities of erythropoietic cells in the bone marrow (Abdalla *et al.*, 1980). Several lines of experimental evidence indicate that erythropoiesis can be severely disrupted by inflammatory mediators such as TNF. *In vitro*, TNF suppresses proliferation of erythroid progenitor cells in human marrow cultures (Roodman *et al.*, 1987). *In vivo*, erythropoiesis is inhibited by chronic exposure to TNF, achieved by implanting nude mice with TNF-secreting CHO cells (Johnson *et al.*, 1989). TNF has been specifically incriminated as a cause of dyserythropoietic anaemia in experimental murine malaria, which recovers

following administration of anti-TNF antibodies (Clark & Chaudhri., 1988; Miller *et al.*, 1989). TNF can also promote erythrophagocytosis, as shown by a transgenic murine model in which TNF was constitutively over-expressed (Taverne *et al.*, 1994).

In a large case-control study in Gambian children, the HLA class I allele, HLA-B53, and the class II allele, HLA-DRB1*1302, have been shown to be associated with protection from severe malarial anaemia (Hill *et al.*, 1991a) These alleles are located 0.25 Mb telomeric and 1 Mb centromeric of the TNF gene respectively. In view of the evidence that TNF may play a role in the pathogenesis of malarial anaemia, we have examined the relationship of severe anaemia to plasma TNF levels and to TNF promoter alleles in the same population of Gambian children.

Subjects and Methods

Children aged up to 10 years were enrolled into a case-control study of severe malaria based at the Royal Victoria Hospital, Banjul, and the Medical Research Council Clinic, Fajara in The Gambia, between 1988 and 1990. Severe malarial anaemia was defined as haemoglobin <5g/dl in association with asexual *P. falciparum* parasitaemia of ≥2500/ul. Cerebral malaria was defined as a Blantyre coma score <3 (persisting for >30 minutes after effective treatment of hypoglycaemia or convulsions) or repeated prolonged seizures (>30 minutes) in a child with *P. falciparum* parasitaemia and no other apparent cause of fits or coma.

The severe malaria group was matched to two control groups for age and area of residence around Banjul. The primary control group was children who were being treated as outpatients for minor illnesses other than malaria, predominantly respiratory and gastrointestinal infections ('mild non-malaria' control group). The second group was children with an uncomplicated febrile illness associated with asexual *P. falciparum* parasitaemia ('mild malaria' control group). Further details of the case-control study are reported elsewhere (Hill *et al.*, 1991a; Kwiatkowski *et al.*, 1990).

Laboratory methods

Plasma TNF levels were measured by enzyme linked immunosorbant assay (ELISA), and the TNF_{-308} A allele was typed by PCR and allele-specific oligonucleotide probing, both as previously described (Kwiatkowski *et al.*, 1990; Chapter II).

The more recently defined TNF promoter variant, a G-A transition at position -238 nucleotides relative to the transcriptional start site (D'Alfonso & Richiardi, 1994) was typed by amplification refractory mutation system PCR using a conserved primer 5'-CCGGATCATGCTTTCAGTGC-3' together with sequence-specific primers for either the TNF_{-238} G allele(5'-AGACCCCCCTCGGAATCG-3') or the TNF_{-238} A allele (5'-AAGACCCCCCTCGGAATCA-3') to generate 459 and 460 base-pair products respectively. As a positive control, each reaction mixture contained the primers 5'-CCAAAGATTCAGGTTTACTCACG-3' and 5'-ACTTAACTATCTTGGGCTGTGAC-3' to amplify a 266 base-pair fragment of the beta 2 microglobulin gene (Gessow *et al.*, 1987). Each 25ul reaction mixture contained approximately 100ng of genomic DNA, 67mM Tris-HCl, 16mM $(NH_4)_2SO_4$, 2mM $MgCl_2$, 100uM of each dNTP (Boehringer Mannheim, Germany), 0.01% Tween-20, 0.5 units of Taq DNA polymerase (Bioline, UK), 0.1uM of each TNF primer and 0.2uM each beta 2 microglobulin primer. The mixture was incubated at 95°C for 10 minutes, followed by 5 cycles of 95°C for 1 minute, 67°C for 1 minute, 72°C for 1 minute, then 25 cycles of 95°C for 1 minute, 62°C for 1 minute, 72°C for 1 minute then a final 10 minutes at 72°C. The products were resolved in 2% agarose gel, stained with ethidium bromide and visualised under UV light.

Direct DNA sequencing of the PCR product was used to confirm the validity of the typing methods. Following purification through a microcon 100 column (Amicon, USA), PCR amplified genomic DNA was cycle sequenced using the ABI PRISM dye terminator kit (Perkin Elmer, USA) using primer 5'-TTTCCTGAGGCCTCAAGCCT-3' and analysed on an ABI377 automated sequencer.

HLA types were initially determined serologically (class I) or by restriction fragment length polymorphism using Taq1 digestion with DRB- and DQB-specific probes (class II). Further analysis of HLA types was done by allele-specific oligonucleotide hybridisation of PCR

products following generic amplification of either the HLA-A, B and C region or the HLA-D region (Wordsworth *et al.*, 1990).

Statistical analysis

Continuous data were compared by Mann Whitney U test. Clinical groups were otherwise compared by X^2 test, using stratified Mantel-Haenszel analysis to correct for the possible confounding effects of ethnic heterogeneity and linkage with HLA class I and II alleles.

Ethical approval

All aspects of the study were approved by the Gambia Government / Medical Research Council Joint Ethical Committee.

Results

Table 8 compares the clinical profile and plasma TNF levels of children with severe malarial anaemia, cerebral malaria or mild malaria. Data are presented on a subset of the total study population as plasma TNF was measured only for those subjects from whom a blood sample was taken immediately after admission to hospital. We found no significant difference in the plasma TNF levels in children presenting with severe malarial anaemia compared to those with mild malaria. This contrasts with our previous finding of elevated plasma TNF levels in Gambian children with cerebral malaria (Kwiatkowski *et al.*, 1990).

With regard to the presenting features of children in each clinical category, for simplicity, children who satisfied the case definition for both cerebral malaria and severe malarial anaemia are omitted from the comparison (table 8). The median age of children with severe malarial anaemia was lower than those with cerebral malaria ($P<0.001$) or with those with mild malaria ($P<0.001$). The severely anaemic group reported a significantly longer duration of symptoms than children with uncomplicated malaria fever ($P<0.001$) or cerebral malaria ($P<0.001$). A larger proportion of children with severe malarial anaemia were found to have splenic enlargement ($P<0.001$) and the geometric mean parasitaemia level on presentation was lower in the anaemic group compared with those with mild malaria ($P<0.001$).

The TNF_{-238} A allele was found in Hardy-Weinberg equilibrium at a frequency of approximately 0.06 in both of the control groups of Gambian children. We found an excess of the TNF_{-238} A allele in children with severe malarial anemia when these were compared to either the non-malaria control group (odds ratio 1.7; 95% CI 1.02-2.8; P= 0.04, Mantel-Haenszel X^2 weighted for ethnic group) or to the mild malaria control group (odds ratio 1.9; 95% CI 1.1-3.1; P= 0.02) (Table 9). We found no association of the TNF_{-238} A allele with cerebral malaria, which contrasts with our previous finding for the TNF promoter TNF_{-308} A allele, where we reported an association of the TNF_{-308} A homozygous state with cerebral malaria but not severe malarial anaemia (Chapter II). The TNF_{-308} A and TNF_{-238} A alleles were not linked in this population and the relationship between the TNF_{-238} A allele and severe anaemia was unaffected by stratification for the TNF_{-308} genotype.

On analysis of associations between the TNF_{-238} genotype and alleles in the HLA class I and class II regions, we found the TNF_{-238} A allele to be in linkage disequilibrium with HLA-B53 (OR=3.9, $P < 10^{-7}$), an unexpected result since HLA-B53 is known to be associated with protection against severe malarial anaemia in this population (Hill *et al.*, 1991a). Correction for HLA-B53 status served to strengthen the association between the TNF_{-238} A allele and susceptibility to severe malarial anaemia (comparing severe malarial anaemia group with non-malaria control group, odds ratio rises from 1.7 to 2.5; P=0.0006. Mantel-Haenszel X^2 stratified for presence or absence of HLA-B53). Conversely the relationship between HLA-B53 and protection from severe malarial anaemia was strengthened by correction for TNF_{-238} genotype (odds ratio falls from 0.42 to 0.32; P=0.00004, Mantel-Haenszel X^2 stratified for presence or absence of TNF_{-238} A).

HLA-DRB1*1302, which is associated with protection against severe malarial anaemia in The Gambia (Hill *et al.*, 1991a) was found to be negatively linked to the TNF_{-238} A allele (odds ratio 0.46, P=0.0002). Linkage disequilibrium was also shown for HLA-B18 (odds ratio 17.9, $P<10^{-7}$) and HLA-DR9 (odds ratio 7.1, $P<10^{-7}$), neither of which is associated with severe malaria in The Gambia. Stratification for these alleles did not affect the association of the TNF_{-238} A allele with severe anaemia.

Table 8. Clinical features and laboratory investigations of Gambian children presenting with malaria of different severity. Continuous data are presented as mean value, or geometric mean for parasitaemia, with 95% confidence intervals in parenthesis.

	mild malaria	cerebral malaria alone	severe malaria anemia alone
n	191	166	63
Hb (g/dl)	8.7 (8.4-9.0)	8.3 (8.0-8.6)	4.3 (4.0-4.6)
age (years)	4.2 (4.0-4.4)	4.1 (3.9-4.3)	2.2 (2.0-2.4)
duration of symptoms (days)	3.7 (3.3-4.1)	2.7 (2.4-3.0)	5.0 (4.1-5.9)
spleen >2cm (% of cases)	11%	14%	32%
parasitaemia ($x10^9$/L)	59(47-73)	64 (44-93)	30 (17-54)
TNF (pg/ml)	18 (15-21)	57 (43-78)	13 (8-20)

Table 9. Distribution of the TNF $_{-238}$ A allele and the TNF $_{-238}$ - HLA-B53 haplotype by clinical condition. Sixty-one individuals satisfied diagnostic criteria for both cerebral malaria and severe malarial anaemia. These are included in the severe malarial anaemia group for the analyses presented in the table and text.

	non-malaria controls	mild malaria controls	cerebral malaria (alone)	severe malarial anaemia (all)
n	371	349	323	193
TNF $_{-238}$ A heterozygotes	45 (12.1%)	38 (10.9%)	43 (13.3%)	36 (18.7%)
TNF $_{-238}$ A homozygotes	3 (0.8%)	2 (0.6%)	2 (0.6%)	2 (1.0%)
Haplotypes:				
B53+ TNF $_{-238}$ A +	27 (7.3%)	22 (6.3%)	13 (4.0%)	19 (9.8%)
B53+ TNF $_{-238}$ A -	71 (19.1%)	65 (18.6%)	44 (13.6%)	10 (5.2%)
B53- TNF $_{-238}$ A +	20 (5.4%)	18 (5.2%)	31 (9.6%)	19 (9.8%)
B53- TNF $_{-238}$ A -	252 (67.9%)	243 (69.6%)	228 (70.6%)	141 (73.1%)
B53 not typed	1	1	7	4

Discussion

The pathogenic process of severe malarial anaemia is complex and incompletely understood. In many cases, the clinical features are consistent with prolonged infection due to poorly developed anti-malarial immunity. Our hypothesis is that chronic low-grade production of TNF, in response to *P.falciparum* parasitaemia, induces dyserythropoiesis and contributes to the pathogenesis of malarial anemia. We have found plasma TNF levels not to be elevated in children presenting with severe malarial anemia compared to those with uncomplicated malaria fever. However, because of the relative chronicity of the illness, as compared to the typically acute presentation of cerebral malaria, and the short circulating half-life of TNF, standard plasma TNF measurements may be expected to shed little light on the question of whether severe anemia might result from excessive TNF production. Since it is extremely difficult to quantitate the production rates of TNF by plasma measurements, and since it is not feasible to obtain bone marrow histology on the majority of patients, we opted to address this problem by examining for associations of severe malarial anemia with polymorphic markers in the TNF promoter region.

We found the TNF_{-238} A allele to be associated with susceptibility to severe malarial anemia, but not to cerebral malaria. In contrast, in the same population, we have previously found the neighboring, TNF_{-308} A allele to be associated with cerebral malaria, but not with severe malarial anemia (Chapter II). Taken together, these findings suggest that the clinical outcome of malaria is influenced by complex genetic determinants in the neighborhood of the TNF gene. Linkage disequilibrium of the TNF_{-238} A allele with several HLA alleles was identified but none of these account for the association with susceptibility to severe malarial anemia. Of particular note, we found the TNF_{-238} A and HLA-B53 alleles to be linked but their effects on susceptibility to severe malarial anemia work in opposite directions. This raises the intriguing possibility that in this case natural selection has favored a haplotype that balances the risks of one immunological characteristic against the benefits of another.

The location of the TNF_{-238} A allele in the TNF promoter region raises the possibility that it could influence constitutive TNF production directly (D'Alfonso & Richiardi, 1994).

However, a study of the TNF_{-238} A allele at the cellular level failed to reveal an effect on TNF response to short term stimulation with bacterial lipopolysaccharide (Pociot *et al.*, 1995). Thus, as may be the case with the TNF_{-308} A allele, the TNF_{-238} A allele may serve as a marker for a functional polymorphism elsewhere in the TNF gene or in another nearby immunologically relevant gene. As TNF transcriptional regulation is known to be cell-type and stimulus specific (Tsai *et al.*, 1996), further studies are required to determine the true functional importance of the allele in the context of chronic stimulation by *P. falciparum*.

These findings in malaria relate to a general problem in the pathogenesis of infectious disease. There is clear evidence that TNF mediates acute symptoms such as fever and septic shock. From experimental evidence it appears probable that TNF also contributes to the cachexia and anemia of protracted infection and this finding of an association between a polymorphic marker in the TNF promoter region and malarial anemia lends weight to this hypothesis. Further association studies in genetically distinct populations and studies in family groups would help to define precisely the genetic elements involved.

IV

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

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Transactions of the Royal Society of Tropical Medicine and Hygiene, **92**, 50-53.

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Summary

The pyrogenic cytokine, 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 compared to 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.

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 TNF and other pyrogenic cytokines (Kwiatkowski *et al.*, 1989; 1993). However, elevated levels of TNF have been detected in the plasma of children with *P. 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, and 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 two cell-surface receptors for TNF also exist as soluble forms, termed sTNF-R55 and sTNF-R75, and these 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 considers 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.*, 1995c 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 (1mg/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 treated appropriately. A second thick film was stained with Giemsa's stain and 100 microscope fields were examined (x1000); parasite density was estimated assuming that one parasite per field is equivalent to 500/ul (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 TNF 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 coded 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 0.2ng/ml and 2ng/ml respectively. The antibodies were the gift of Celltech Limited, UK.

Assays were performed on samples subjected to no more than two freeze-thaw cycles.

Statistical analysis

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

Ethical approval

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 and no other obvious cause of fever (P+F+). The second category was 120 children (38.3%) who were afebrile despite infection with *P.falciparum* (P+F-). The third category was 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 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/ul, 95% confidence interval 5901- 36421) than in the P+F- group (610/ul, 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. 2).

Geometric mean levels of TNF were higher in the P+F+ group (51pg/ml) than in the P+F- group (21pg/ml; $P<0.001$), and higher in the P+F- group than in the P- group (21pg/ml versus 15pg/ml; $P = 0.003$). In contrast, neither sTNF-R55 nor sTNF-R75 levels differed significantly among the 3 clinical categories (Fig.3).

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). Importantly, 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).

Fig. 2. Mean temperature and geometric mean levels of TNF, sTNF-R55 and sTNF-R75 in relation to parasite density. 173 children had no parasites, 122 had <5000 parasites/ul, and 18 had ≥5000 parasites/ul. Error bars represent 95% confidence intervals.

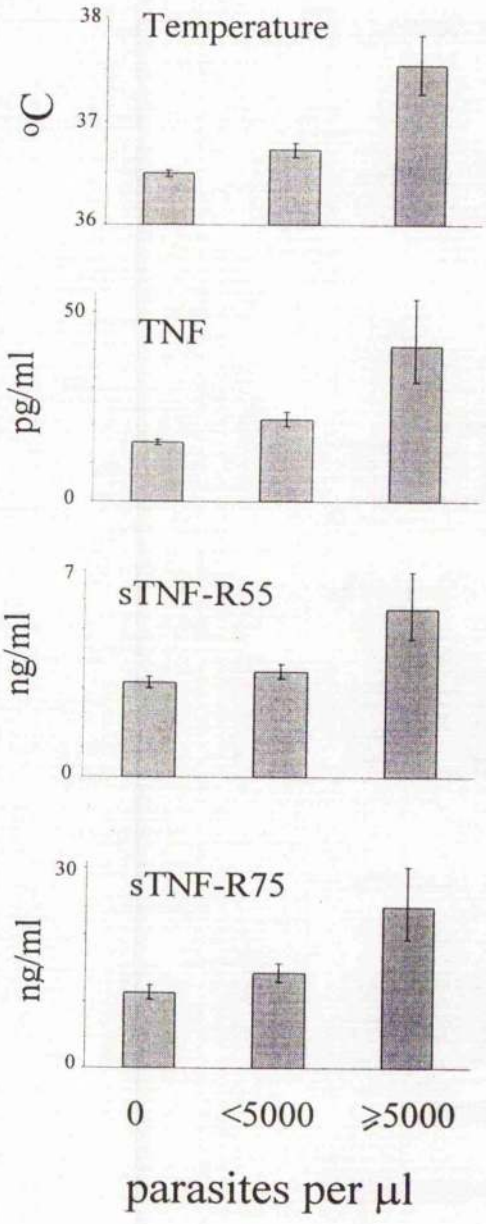
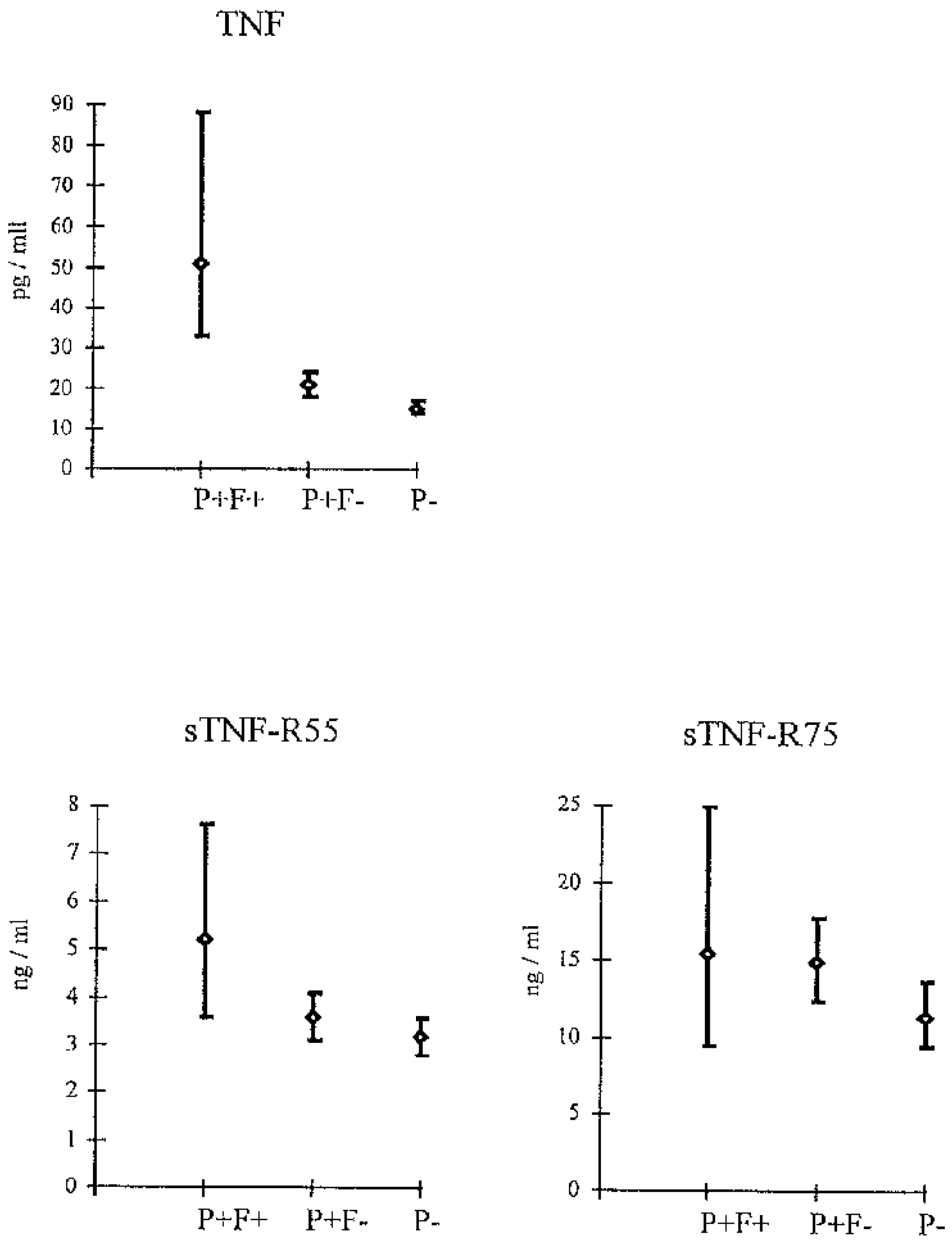


Fig. 3. Geometric mean levels of TNF, sTNF-R55 and sTNF-R75 in relation to clinical category. 173 children had no parasites (P-), 120 had parasitaemia but were afebrile (P+F-), and 20 had parasitaemia with fever (P+F+). Error bars represent 95% confidence intervals.



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.

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 *et al.*, 1993; Boele van Hensbrock *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 contribute 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 are significantly higher in children who are both parasitaemic and febrile at the time of examination, compared to those who are 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 in the plasma, yet the data provide 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 in those who were febrile compared to 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 crossreact 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 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 effect 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 two 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, prior to 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.

V

Circulating ICAM-1 levels in falciparum malaria are high but are unrelated to disease severity

[1]McGuire, W., [2]Hill, A.V.S., [3]Greenwood, B.M. & [1]Kwiatkowski, D. (1996).
Transactions of the Royal Society of Tropical Medicine and Hygiene, **90**, 274-276.

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Summary

Intercellular adhesion molecule-1 (ICAM-1) mediates the binding of *Plasmodium falciparum* to vascular endothelium. In a case-control study of falciparum malaria in Gambian children, we have looked for evidence that a generalised increase in expression of ICAM-1 is associated with cerebral malaria. Plasma levels of circulating ICAM-1 (cICAM-1) were significantly higher in 246 children with acute malaria than in 156 children with non-malarial illnesses. cICAM-1 levels correlated with levels of TNF, interleukin-1 alpha (IL-1) and interferon-gamma, supporting the view that these cytokines are responsible for a general up-regulation of ICAM-1 expression in malaria. However, while it has been previously shown that TNF and IL-1 levels were related to disease severity, this was not the case for cICAM-1. It may be that differences in the distribution of ICAM-1, rather than its total level of expression, are critical in determining the clinical outcome in malaria.

Introduction

A major pathological feature of falciparum malaria is the sequestration of parasitised erythrocytes (PRBC) in small blood vessels (MacPherson *et al.*, 1985; Turner *et al.*, 1994). Cerebral malaria is believed to be a consequence of this phenomenon occuring in post-capillary venules in critical areas of the brain. Sequestration is the result of a specific interaction between ligand(s) expressed on the surface of PRBC and receptors expressed on vascular endothelium (Berendt *et al.*, 1990). One of the best characterised of these endothelial receptors is intercellular adhesion molecule-1 (ICAM-1) (Berendt *et al.*, 1989). ICAM-1 is a membrane glycoprotein whose physiological role is to mediate leucocyte adhesion by acting as a receptor for two ligands which are variably expressed on the leucocyte surface, namely leucocyte function antigen 1 (LFA-1, CD11a/CD18) and Mac-1 (CD11b/CD18). The binding site for parasitised erythrocytes is distinct from those of the leucocyte integrins (Berendt *et al.*, 1992; Ockenhouse *et al.*, 1992b).

Expression of ICAM-1 on human vascular endothelium can be up-regulated by the pro-inflammatory cytokines TNF and IL-1 (Dustin *et al.*, 1986). These cytokines are present at high levels in the plasma of African children with malaria and elevated levels are related to

disease severity and outcome (Kwiatkowski *et al.*, 1990). Several investigators have pointed out that high levels of TNF and IL-1 production may exacerbate parasite sequestration by up-regulating ICAM-1 expression on endothelium and that this may be a critical step in the pathogenesis of cerebral malaria (Berendt *et al.*, 1990). However, it has proved difficult to explore this hypothesis *in vivo*.

There are obvious difficulties in obtaining a direct measurement of ICAM-1 expression in the blood vessels of children with malaria but an indirect measure of ICAM-1 expression may be provided by the amount of soluble ICAM-1 that is shed into the circulation. Studies *in vitro* have shown that the release of soluble ICAM-1 correlates with levels of ICAM-1 expressed on endothelium in response to TNF and IL-1 (Leeuwenberg *et al.*, 1992). Soluble (circulating) forms of ICAM-1 (cICAM-1) have been detected in the plasma of patients with a variety of inflammatory disease states (Gearing & Newman, 1993), including acute falciparum malaria (Hviid *et al.*, 1993; Deloron *et al.*, 1994; Graninger *et al.*, 1994; Jakobsen *et al.*, 1994).

The present study was designed to test the hypothesis that cerebral malaria in African children might be related to a generalised increase in ICAM-1 expression, consequent on high levels of TNF and IL-1 production.

Patients and Methods

We studied Gambian children, aged 1-10 years, who attended the Royal Victoria Hospital, Banjul or the Medical Research Council (MRC) Laboratories, Fajara in the 1988 rainy season. As part of a large case-control study of cerebral malaria in this population (described in detail in Kwiatkowski *et al.*, 1990), venous blood samples were collected from 157 children who presented with cerebral malaria (*Plasmodium falciparum* parasitaemia, Blantyre coma score <3, no other cause of coma), of whom 132 survived the episode and 25 died; 89 children with mild malaria (*P. falciparum* parasitaemia, axillary temperature > 37.4°C, no other cause of fever); and 156 children with a variety of infectious diseases other than malaria (mainly acute respiratory tract and gastrointestinal infections of varying degrees of severity, no malaria parasites seen on a thick blood film).

Plasma (with ethylenediaminetetraacetatic acid and aprotinin) was separated within one hour of collection and stored frozen at -20°C until assayed. TNF, IL-1 and interferon-gamma (IFN) were assayed as described previously (Kwiatkowski *et al.*, 1990).

cICAM-1 was measured by an enzyme linked immunosorbent assay (British Bio-technology Limited, Oxford, UK). Briefly, plasma samples diluted 1:100 in phosphate buffered saline were added to polystyrene plates precoated with anti-ICAM-1 mouse monoclonal antibody. A biotinylated anti-ICAM monoclonal antibody was added and, after incubation at room temperature for 2 hours and extensive washing, streptavidin conjugated with horseradish peroxidase was added. After further incubation for hour followed by washing the reaction product was developed in tetramethyl-benzidine substrate solution for 10 minutes, after which the reaction was terminated with 2N sulphuric acid. The optical density (absorbance) at 450nm of the developed product was assayed and cICAM levels in samples were estimated from the standard curve for each assay. The detection limit was 2.5ng/ml and, for statistical purposes, results below this limit were assigned values of 1.25ng/ml.

Ethical approval

Ethical approval was obtained from the Gambian Government / MRC Joint Ethical Committee.

Results

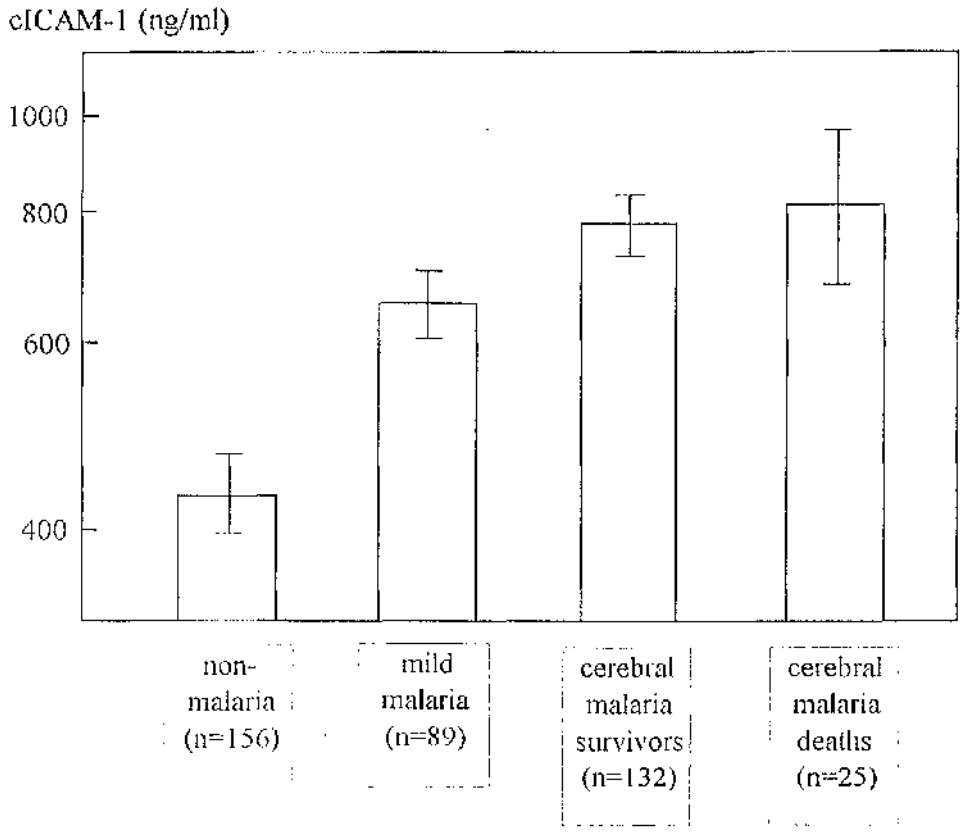
The median age of children who survived the episode of cerebral malaria was 3.8 years and that of those who died was 3.0 years. The median ages in the mild malaria and non-malaria groups were 3.4 years and 2.2 years respectively.

Levels of cICAM-1 correlated with those of IL-1 (Spearman rank correlation coefficient, r=0.29, P<0.001), TNF (r=0.29, P<0.001) and IFN (r=0.24, P<0.001). Since the plasma levels of the cytokines were correlated with each other, we performed a multiple linear regression analysis to determine if each cytokine independently correlated with cICAM-1. In this model, both IL-1 (P=0.01) and TNF (P=0.04), but not IFN, were significant predictors of cICAM-1 levels.

Comparing the group of children with acute malaria (mild and cerebral) with those with non-malarial illness, the geometric mean cICAM-1 level was higher in the malaria group (735ng/ml, 95% confidence interval (CI) 689-810ng/ml versus 440ng/ml, 95% CI 395-493; $P<0.001$, by t-test on log-transformed variables). To determine whether this association between cICAM-1 with malaria was independent of the measured cytokines, we did a logistic regression analysis of children with malarial versus non-malarial illnesses against TNF, IL-1, IFN and cICAM-1 (all variables log-transformed). The most significant correlates of malaria were the levels of TNF ($P<0.001$) and cICAM-1 ($P<0.001$).

We found no significant difference in geometric mean cICAM-1 levels in children presenting with cerebral malaria (779ng/ml, 95% CI 704-861ng/ml) compared to those with mild malaria (685ng/ml, 95% CI 584-804ng/ml). Similarly, there was no significant difference in cICAM-1 levels between those children who survived an episode of cerebral malaria compared with those who died (775ng/ml, 95% CI 694-865ng/ml versus 801ng/ml, 95% CI 585-1097ng/ml respectively). (Fig. 4). These findings for cICAM-1 are in contrast to our findings for TNF (geometric mean level in mild malaria = 10pg/ml, in cerebral malaria = 19pg/ml, and in children who die of cerebral malaria = 99pg/ml) and IL-1 (10pg/ml, 17pg/ml and 40pg/ml respectively) in this sample.

Figure 4. Circulating ICAM-1 level (cICAM-1) in Gambian children presenting with a variety of clinical conditions; columns indicate geometric means (logarithmic scale), vertical bars indicate +/- one standard error.



Discussion

It is not known why only a minority of African children infected with *Plasmodium falciparum* develop cerebral malaria (Greenwood *et al.*, 1991). Parasite virulence factors, such as cytoadherence phenotype (Roberts *et al.*, 1992), may be important, although to date only erythrocyte rosetting has been demonstrated to be associated with disease severity (Carlson *et al.*, 1990). Host factors may also be involved and there has been considerable interest in the role of the host cytokine response in determining the clinical outcome of infection. In this population, the plasma levels of TNF and IL-1 have been shown to be independent predictors for cerebral malaria and specifically for a fatal outcome from this condition (Kwiatkowski *et al.*, 1990). Recently we have demonstrated that Gambian children with a genetic propensity to produce higher amounts of TNF are at increased risk of developing and dying from cerebral malaria (Chapter II). Since TNF and IL-1 upregulate expression of ICAM-1, it has been suggested that one way that these cytokines contribute to the pathophysiology of cerebral malaria is by mediating enhanced sequestration of PRBC (Berendt *et al.*, 1990).

Because of the problems involved in obtaining appropriate post-mortem tissue samples it has proved difficult to examine the role of endothelial receptor expression in the pathogenesis of cerebral malaria in African children. We have therefore attempted to examine the relationship between cytokine and ICAM-1 expression in this population by measuring the level of circulating plasma ICAM-1 in children recruited to a case-control study of cerebral malaria in The Gambia.

We have found circulating ICAM-1 levels to correlate with plasma levels of the pro-inflammatory cytokines, TNF and IL-1, supporting *in-vitro* evidence that these cytokines induce enhanced expression and shedding of ICAM-1 (Dustin *et al.*, 1986; Leeuwenberg *et al.*, 1992). cICAM levels were found to be significantly higher in Gambian children with falciparum malaria than in comparable children with non-malarial illnesses. If cICAM-1 levels in plasma do reflect cell-surface expression, as is the case *in vitro*, then these data support previous immunohistochemical evidence of a cytokine mediated upregulation of ICAM-1 expression in acute malaria (Turner *et al.*, 1994).

Unlike a previous, smaller study in The Gambia which found higher levels of cICAM-1 in children with severe malaria (severe malaria anaemia and cerebral malaria) compared to children with mild malaria (Jakobsen *et al.*, 1994), these data provide no evidence that the overall level of ICAM-1 expression in itself is critical in determining the clinical outcome of *P.falciparum* infection since we have found cICAM-1 levels not to differ between children with cerebral malaria and those with mild malaria. Deloron *et al.* (1994) also found no difference in the cICAM-1 levels in Burundian adults with cerebral malaria compared to those with severe malaria without cerebral involvement, and concluded that ICAM-1 was not involved in the pathophysiology of cerebral malaria. Although our data are consistent with this interpretation, it may be that when levels of ICAM-1 expression are generally high, as in malaria, small regional differences in expression may critically affect the site of maximum PRBC sequestration. The tissue distribution of ICAM-1 expression, rather than the total level of expression, may be the more important determinant of whether an individual child develops cerebral malaria. Resolving this issue requires detailed post-mortem comparisons between patients dying of cerebral and of non-cerebral malaria.

Although elevated plasma cICAM-1 in acute malaria may simply reflect receptor shedding or de-adherence of sequestered PRBCs (or activated lymphocytes) from endothelium, an intriguing question is whether these circulating forms are of functional significance in disease. A number of other cell-surface receptors have been shown to exist in a cleaved soluble form in body fluids, but in only a few cases has a functional role been defined. *In vitro*, the soluble form of ICAM-1 has been shown to modulate ICAM-1 mediated cell-cell interactions (Becker *et al.*, 1991), and release of ICAM-1 in the upper respiratory tract may inhibit rhinovirus infection (Marlin *et al.*, 1990). The release of cICAM-1 and other vascular adhesion molecules may be important in cell-signalling, chemo-attraction or adhesion blocking (Gearing & Newman, 1993). Soluble ICAM-1, at concentrations lower than we have detected in the plasma of children with malaria, has been shown to inhibit PRBC binding to ICAM-1 in an *in vitro* binding assay (Ockenhouse *et al.*, 1992b), raising the possibility that the high level of cICAM-1 in malaria competitively modulates PRBC binding to endothelium *in vivo*.

VI

C-reactive protein and haptoglobin in the evaluation of a community based malaria control programme

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Transactions of the Royal Society of Tropical Medicine and Hygiene, 90, 10-14.

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Summary

When cross-sectional surveys are used to evaluate malaria intervention programmes in the community, the prevalence of morbidity is difficult to assess because of the fluctuating nature of malarial fever. We have therefore investigated the impact of bed net usage on two surrogate markers of malarial morbidity: (i) elevated C-reactive protein (CRP) (>8mg/l) plus detectable parasitaemia, as an indicator of malaria-induced acute-phase response; and (ii) reduced haptoglobin levels (<180mg/l) which in this population indicates malaria-induced intravascular haemolysis. Among 1505 Gambian children of 1-5 years old, examined on a single occasion at the end of the malarial transmission season, 5% had parasitaemia plus fever, while 24% had parasitaemia plus elevated CRP, and 35% had low haptoglobin. The proportion of children who had parasitaemia plus elevated CRP was significantly lower than in those who had slept under insecticide-treated bed nets compared to those who did not use a bed net (16% *vs* 34%, $P<0.003$), and the proportion with a low haptoglobin differed similarly (24% *vs* 49%, $P<0.003$). Use of an untreated bed net had a weaker effect on both indices (22% had parasitaemia plus elevated CRP, 34% had low haptoglobin). CRP and haptoglobin are simple and inexpensive to measure in large numbers of people, and these results suggest that they could be useful for the assessment of malaria intervention programmes.

Introduction

It is important to evaluate the impact of malaria control strategies on the level of malaria-specific morbidity and mortality in the community. This is best determined by a period of longitudinal monitoring but, in rural African communities, this can be logistically complex and prohibitively costly. In general, estimates of the level of malaria morbidity rely on cross-sectional surveys of a sample of the population. In endemic regions, however, malaria illness is not indicated solely by the presence of parasitaemia as many *Plasmodium falciparum* infections, even at high density, are asymptomatic (Marsh, 1992). Disease episodes can be defined as parasitaemia associated with fever, and this case definition is appropriate for use in longitudinal studies (Snow *et al.*, 1989). However, since malaria fever is typically intermittent, this definition of malaria illness is inappropriate for use in

cross-sectional surveys as disease episodes will be under-detected. The other traditional malariometric indices, spleen rate (the proportion with a palpably enlarged spleen) and prevalence of anaemia, are also of limited value as cross-sectional measures since they change only slowly in response to effective malaria control (Rougemont *et al.*, 1988).

An awareness of the limitations of the standard malariometric indices has led to the suggestion that stable biochemical markers of disease activity may be better cross-sectional measures of the level of malaria illness in a community (Rougemont *et al.*, 1988; Sisay *et al.*, 1992; Hurt *et al.*, 1994). The main requirement of such a marker is that it reflects disease activity rather than simply parasitaemia.

A potentially useful marker of disease activity is C-reactive protein (CRP). This is an acute phase protein, generated in response to pyrogenic cytokines (TNF, interleukins 1 and 6) which host monocytes and macrophages secrete when malaria schizonts rupture (Kwiatkowski *et al.*, 1989; Karunaweera *et al.*, 1992). These cytokines have a short half-life in plasma and therefore, like fever, are not useful as cross-sectional epidemiological markers. Several studies have demonstrated increased plasma CRP levels in African children with malaria (Ree, 1971; Naik & Voller, 1984; Hurt *et al.*, 1994). When malaria fever has remitted in patients treated for falciparum malaria plasma CRP remains elevated for several days (unpublished observation). CRP may therefore be a more stable marker of disease activity than is fever. In the same way that elevated body temperature associated with parasitaemia defines a clinical malaria episode in longitudinal surveys, an elevated plasma CRP level associated with parasitaemia may be a better index of malaria illness, for use in cross-sectional surveys, than is parasitaemia alone.

Another possible marker is haptoglobin. Although this is an acute phase protein, its expression is not increased to the same extent as is CRP in response to pyrogenic cytokines (Stadnyk & Gauldie, 1991), and the principal effect of malaria parasitaemia on haptoglobin relates to intravascular haemolysis. Haptoglobin binds and clears free haemoglobin and therefore haemolysis results in reduced plasma haptoglobin levels. Haemolysis secondary to malaria is the only significant cause of hypohaptoglobinaemia in African populations (Trape *et al.*, 1985). It has been proposed that the distribution of haptoglobin levels may be useful

in the evaluation of malaria control programmes (Rougemont *et al.*, 1988; Trape & Fribourg-Blanc, 1988; Sisay *et al.*, 1992).

The aim of this study was to investigate the usefulness of CRP and haptoglobin as surrogate markers of malaria morbidity and to define their relationship to conventional malariometric indices. Our strategy was to determine how CRP and haptoglobin levels were affected by bed net usage which, in several studies in The Gambia, has been shown to reduce malaria morbidity and mortality (Snow *et al.*, 1988a, 1988b; Alonso *et al.*, 1991; D'Alessandro *et al.*, 1995a, 1995c).

Subjects and Methods

In June 1992, the Gambian government implemented a national impregnated bed net programme (NIBP) in about half of all the primary health care (PHC) villages in the country. The aim was to treat all of the bed nets in the selected villages with the insecticide permethrin. PHC villages usually have a population of greater than 400 and support a resident village health worker and traditional birth attendant. During the subsequent transmission season (August-December 1992) the Medical Research Council longitudinally monitored the impact of the programme on malaria mortality (See D'Alessandro *et al.*, 1995a for details).

In addition to the longitudinal assessment, a cross-sectional clinical survey of a sample of the population was undertaken at the end of the 1992 rainy season (October and November). During this survey, we randomly selected 1505 Gambian children (aged 1-4 years) from villages from each of the 5 NIBP surveillance areas (the areas are described by Thomson *et al.*, 1994 and D'Alessandro *et al.*, 1995a). The children were examined, their axillary temperature measured and spleen size estimated, and a finger-prick blood sample was taken for parasitological examination, PCV measurement and CRP and haptoglobin assay. The blood was collected into a tube containing sodium ethylenediaminetetraacetate (1mg/ml) and aprotinin (0.5 trypsin inhibitor unit/ml) and plasma was separated immediately by centrifugation at 10000 *g* and stored frozen until assayed. Further details of the cross-sectional survey are given by D'Alessandro *et al.* (1995c).

CRP determination

Plasma CRP was determined by ELISA, based on the method of Voller *et al.* (1980). Briefly, microtitre plates were coated with rabbit anti-human CRP polyclonal antibody (Dako) in Tris-HCl. After washing, samples diluted 1:10000 in phosphate buffered saline and CRP standards (Dako) in the range 0.8 to 50mg/l were added and the plates incubated at 37^{0}C for 2 hours. After washing, a horse-radish peroxidase (HRP) conjugated anti-CRP antibody (Dako) was added and after 2 hours incubation at 37^{0}C the plates were washed again before o-phenylenediamine dihydrochloride (OPD) (Sigma) in phosphate-citrate buffer was added. The reaction was stopped with sulphuric acid and the optical density at 492nm measured. Under these conditions, the lower limit of detection was 7.8 mg/l and, for statistical purposes, results below this were assigned values of 5mg/l.

Haptoglobin determination

Plasma haptoglobin was also determined by ELISA, based on the method described by Yerly *et al.* (1990). Briefly, microtitre plates were coated with sheep anti-haptoglobin polyclonal antibody (Serotec) in borate buffered saline (BBS). After washing, samples diluted 1:20000 in BBS and standards (Sigma) in the range 0.8 to 50ug/l were added and incubated at 37^{0}C for 2 hours. After washing, an HRP conjugated anti-haptoglobin antibody (Serotec) was added and after 2 hours the plates were washed again before OPD in phosphate-citrate buffer was added. The reaction was stopped with sulphuric acid and the optical density at 492nm measured. Under these conditions, the lower limit of detection was 7.8mg/l and results below this were assigned values of 5mg/l.

Statistical analysis

Two separate comparisons were made: (i) children who did not sleep under bed nets were compared to children who slept under untreated bed nets, and (ii) children who did not use bed nets were compared to children who used insecticide treated nets. This second comparison only included children who lived in PHC villages since insecticide treatment of bed nets was only available in PHC villages. In making comparisons, additional confounding variables were accounted for by stratifying in a Mantel-Haenszel X^2 test.

Ethical approval

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, 1505 children, 50% female, were studied. Mandinka was the most common ethnic group (33%), followed by Fula (20%), Sarahuli (19%), Jola (15%), and Wollof (10%). 770 (51%) children slept regularly under an untreated bed net and 350 (23%) a permethrin treated net, and 385 (26%) did not use a bed net.

Clinical profile and standard malariometric indices

Asexual forms of *P.falciparum* were detected in 570 (38%) children. Of these, 79 (14%) were febrile with an axillary temperature ≥37.5°C. In 210 (37%) of the parasitaemic children the parasite density was ≥5000/ul, and amongst those, 51 (24%) were febrile.

In total, fever was detected in 95 (6.3%) children. Of these febrile children, 79 (83%) had detectable *P.falciparum* parasitaemia and 51 (54%) had parasitaemia ≥5000/ul. A palpably enlarged spleen (Hackett class >0) was detected in 330 (22%) children; 157 (10%) children had significant anaemia (PCV <25%).

The effect of untreated and treated bed net use on these standard malariometric indices has been presented in detail elsewhere (D'Alessandro *et al.*, 1995c) and is summarised in Table 10.

Table 10. Summary of effect of bed net use on standard malariometric indices in the population sample for whom plasma levels of C-reactive protein and haptoglobin were available.

	All villages		PHC villages only	
	No bed net	Untreated bed net	No bed net	Treated bed net
n=	385	770	215	348
Parasite rate	52%	35%	51%	28%
Parasitaemia ≥5000/ul	19%	14%	17%	9%
Spleen rate	31%	21%	28%	16%
Mean PCV (SD)	31.4 (4.9)	32.1 (4.8)	31.9 (4.9)	33.0 (4.4)

Distribution of CRP and haptoglobin within the sample

An elevated CRP, defined as >8mg/l (Young *et al.*, 1991), was detected in 37% of the sample. High CRP associated with *P.falciparum* parasitaemia was found in 24%. High CRP associated with *P.falciparum* parasitaemia ≥5000/ul was detected in 11%.

Plasma CRP levels in this sample were unrelated to sex, age or ethnic group but varied, as did the standard indices such as parasite and spleen rates, between the 5 NIBP surveillance areas. The proportion of children with an elevated CRP was highest in the eastern part of the country in surveillance areas areas 4 and 5 (49% in areas 4-5 compared to 30% in areas 1-3, $X^2 = 52$, $P<0.001$).

A low level of haptoglobin (<180mg/l; Rougemont *et al.*, 1988; Yerly *et al.*, 1990) was found in 35% of the children. As with CRP, plasma haptoglobin levels varied between NIBP surveillance areas. The proportion of children with a low haptoglobin was highest in the two surveillance areas in the east of the country (46% in areas 4-5 compared to 27% in areas 1-3, Mantel-Haenszel X^2 (stratified for age) = 115, P<0.001). Haptoglobin levels were inversely related to age (allowing for area) but unrelated to sex or ethnic group.

Relationship of CRP and haptoglobin to parasitaemia and fever

CRP levels were higher in parasitaemic than in non-parasitaemic children (P<0.001 by Mann-Whitney U test) and CRP and parasite density were correlated in the 570 parasitaemic individuals (Spearman correlation co-efficient (all values log transformed) = 0.4, P<0.001) (Table 11, Fig. 5).

Higher plasma CRP levels were also associated with the presence of fever (P<0.001 by Mann-Whitney U test) and the proportion of children with an elevated CRP was higher in the febrile compared to afebrile groups (81% versus 34%: Mantel-Haenszel X^2 (stratified for parasite density above and below 5000/ul) = 18.7, P<0.001).

Haptoglobin levels also differed significantly between the parasitaemic and aparasitaemic groups (P<0.001 by Mann-Whitney U test) but haptoglobin levels did not differ between those with a parasitaemia ≥5000/ul and those with lower parasite densities (Fig. 6). Similarly, whereas parasite density in infected individuals correlated with CRP, there was no correlation between parasite density and haptoglobin level. Haptoglobin levels do not differ significantly between the febrile and afebrile groups (allowing for parasitaemia and age).

Relationship of CRP and haptoglobin to other clinical indices

An elevated CRP level (>8mg/l) was significantly associated with the presence of palpable splenomegaly (Mantel-Haenszel X^2 (stratified for parasitaemia) = 19.4, P<0.001) and of anaemia (X^2 = 12.0, P<0.001) (Table 11). Hypohaptoglobinaemia (<180mg/l) was associated with splenomegaly (Mantel-Haenszel X^2 (stratified for age and parasitaemia), = 43.5, p<0.001) and anaemia (X^2 = 9.5, P<0.003).

Table 11. Relationship of plasma C-reactive protein and haptoglobin levels to level of parasitaemia, splenomegaly, low packed cell volume and pyrexia.

	Parasites/ul			Palpable spleen		PCV <25%		Fever >37.4°C	
	0	<5000	≥5000	No	Yes	No	Yes	No	Yes
n=	935	360	210	1175	330	1348	157	1410	95
CRP (mg/l)	7.1 (6.7-7.5)	13 (11.6-14.6)	34.5 (29.1-40.9)	8.7 (8.2-9.1)	18.5 (16.2-21.4)	9.7 (9.2-10.3)	16 (13.3-19.2)	9.4 (8.9-9.9)	35.9 (27.6-46.6)
Haptoglobin (mg/l)	441 (406-480)	103 (87-123)	94 (75-177)	342 (314-372)	84 (70-100)	275 (253-299)	115 (86-155)	258 (238-281)	164 (133-238)
CRP >8mg/l	21%	52%	82%	30%	62%	37%	59%	34%	81%
Haptoglobin >180mg/l	19%	58%	65%	26%	64%	33%	54%	34%	47%

Fig. 5. Frequency distribution of plasma C-reactive protein levels stratified for density of parasitaemia: open bars, <8mg/l; hatched bars, <50mg/l,; solid bars, ≥50mg/l.

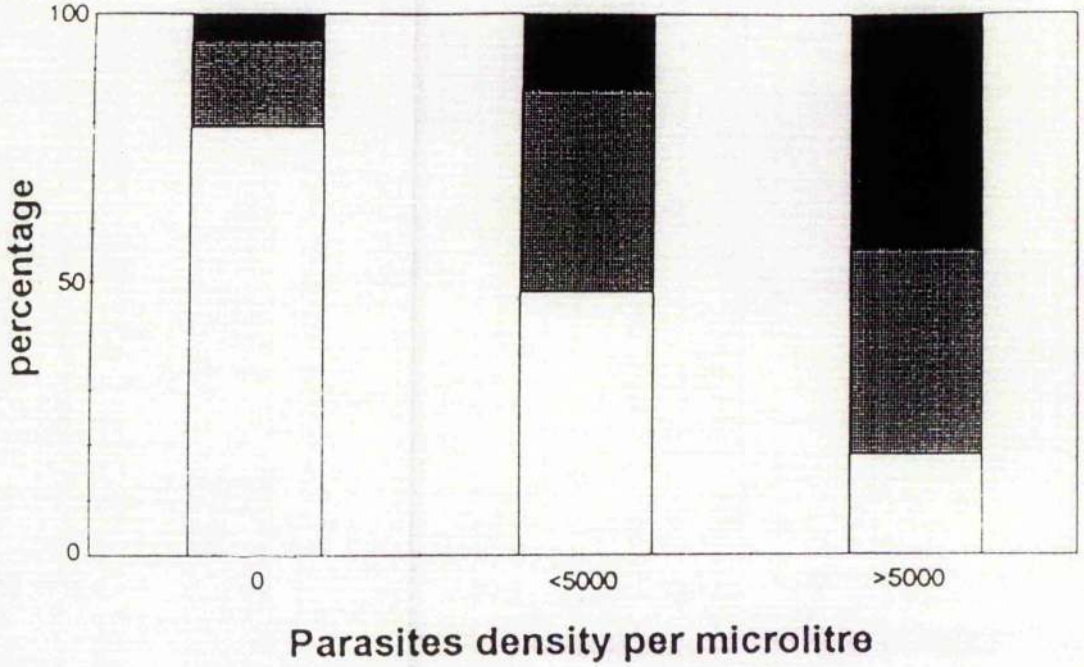
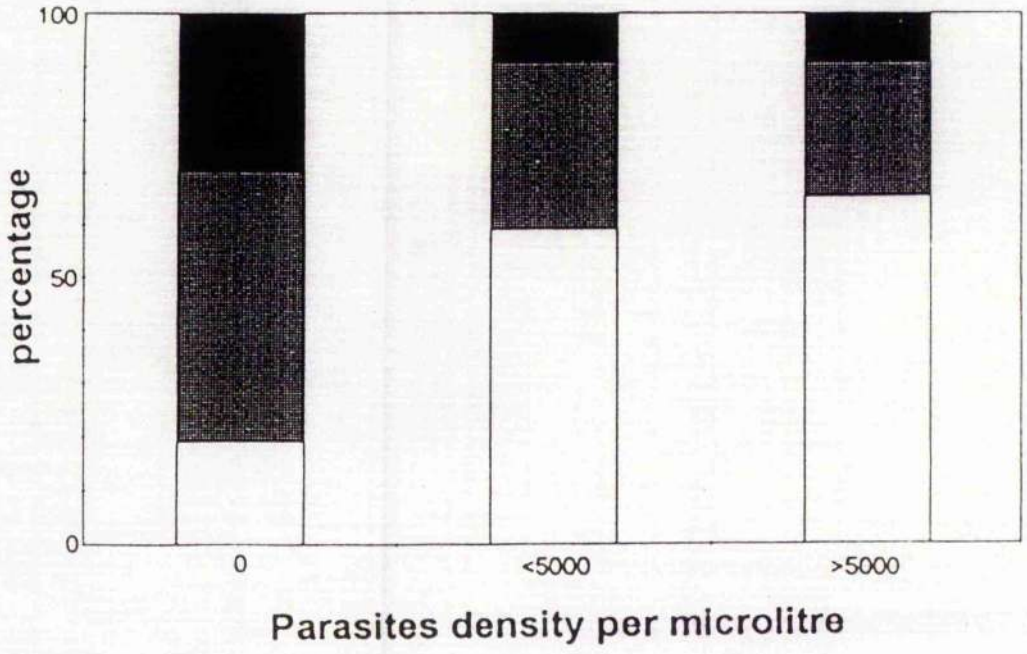


Fig. 6. Frequency distribution of plasma haptoglobin levels stratified for density of parasitaemia: open bars, <180mg/l; hatched bars, <1000mg/l,; solid bars, ≥1000mg/l



Effect of bed net use on CRP and haptoglobin levels

Data for children who did not sleep under a bed net and those who used either a treated or an untreated net are compared in Table 12. To determine if the observed differences in rates of raised CRP and low haptoglobin were due to the effect of bed net use, we allowed for confounding variables and calculated weighted Mantel-Haenszel odds ratios.

A significantly smaller proportion of the group of children who slept under insecticide-treated bed nets had an elevated CRP associated with parasitaemia than did those who did not use a bed net (Mantel-Haenszel X^2 (allowing for both area and age) = 8.9, P=0.003; odds ratio = 0.49, 95% confidence limits (CI) 0.3-0.79). Elevated CRP plus parasitaemia ≥5000/ul was less common in children using treated bed nets than in those not using them, but the difference was not statistically significant. Use of a treated bed net, compared to no net, was associated with a reduction in the proportion of children with a low haptoglobin (Mantel-Haenszel X^2 (allowing for both area and age) = 8.5, P=0.003; odds ratio = 0.53, 95% CI 0.34-0.81). When use of untreated bed nets was compared with no net use, there was a similar trend to that observed with treated bed nets for all of the indices examined, but the trends were not statistically significant.

Table 12. Effect of bed net use on plasma levels of C-reactive protein and haptoglobin.

	All villages		PHC villages only	
	No bed net	Untreated bed net	No bed net	Untreated bed net
n=	385	770	215	348
High CRP				
Any parasitaemia	35%	22%	34%	16%
Parasitaemia ≥5000/ul	16%	11%	15%	7%
Low haptoglobin	45%	34%	49%	24%

Discussion

In this sample of 1505 rural Gambian children, surveyed during the malaria transmission season, 38% had detectable *P.falciparum* parasitaemia but, of these, 86% were apyrexial when examined. Even in the group of children with parasite densities ≥5000/ul, the estimated threshold for fever in The Gambia (Greenwood *et al.*, 1987), 76% were apyrexial when examined. It is likely that a proportion of the afebrile parasitaemic children were febrile in the previous days and/or would have become febrile subsequently. We, and others (Trape *et al.*, 1985), have observed afebrile parasitaemic children who have subsequently become febrile within hours of the original examination. As a consequence of the intermittent nature of malaria fever, the usual clinical case-definition of malaria (elevated body temperature associated with parasitaemia, in the absence of another cause of fever), while appropriate for frequent longitudinal surveillance (Snow *et al.*, 1989), is less useful as a cross-sectional morbidity index. Several workers have therefore suggested that alternative cross-sectional malariometric indices should be considered and evaluated (Rougemont *et al.*, 1988; Marsh, 1992; Hurt *et al.*, 1994).

Both CRP and haptoglobin have been proposed as surrogate measures of malaria morbidity for use both at the individual and the population level (Rougemont *et al.*, 1988; Trape & Fribourg-Blanc, 1988; Gillespie *et al.*, 1991; Sisay *et al.*, 1992; Hurt *et al.*, 1994). We wished to assess these proteins, not to improve diagnostic precision at the individual level but as surrogate markers of malaria morbidity in the community.

A plasma CRP level >8mg/l was found in 37% of the children. CRP levels were more elevated in parasitaemic than in aparasitaemic children and parasite density and CRP level were significantly correlated. Given this correlation, we postulate that malaria is a predominant reason for increased CRP in this population. Elevated CRP was also found in 21% of the aparasitaemic children (Fig. X). Some of these cases may have been due to cleared or sub-patent parasitaemia, since in Gambian children undergoing treated we have noted that plasma CRP levels may remain elevated beyond the time when parasitaemia has been cleared (unpublished observation). However, as infections other than malaria will have accounted for a proportion of the cases of elevated CRP, we coupled the measurement with

parasitaemia to increase the malaria specificity of the index. The proportion of children with an elevated CRP associated with patent parasitaemia was lower in the group who slept under insecticide treated bed nets compared to those without nets. A similar trend was seen with untreated bed net use, but this was not statistically significant. No direct estimate of specificity or sensitivity can be made from this approach, since we have no 'gold standard' cross-sectional definition of malaria illness, but these findings reflect the impact of bed net use on malaria morbidity (monitored longitudinally) and mortality (Snow *et al.*, 1988b; Alonso *et al.*, 1993; D'Alessandro *et al.*, 1995a).

In hyperendemic regions, where up to 90% of children may be parasitaemic at a given time, a problem with this definition (elevated CRP associated with patent parasitaemia) may be low specificity. If the index is defined as elevated CRP combined with high parasitaemia then disease specificity may be improved with little loss of sensitivity (Hurt *et al.*, 1994). We used a cut-off level of 5000 parasites/ul as this has previously been defined as the threshold for fever in The Gambia (Greenwood *et al.*, 1987). In other epidemiological settings, calculation of the fraction of fever cases attributable to malaria at each level of parasite density has been used in the choice of a cut-off value. (Armstrong Schellenberg *et al.*, 1994; Smith *et al.*, 1994). However, only 11% of our total study population were found to have an elevated CRP associated with parasite density ≥5000/ul and therefore the analysis was performed on a smaller sample size than for the other indices examined. Although there was a clear trend, when confounding variables were allowed for, bed net use was not found to significantly affect the proportion of children with this definition.

Intra-vascular haemolysis secondary to malaria is the only significant cause of hypohaptoglobinaemia in African populations (Trape *et al.*, 1985). It is therefore unnecessary to combine a reduced haptoglobin level with the detection of parasitaemia in order to make the index more malaria specific. Although significantly lower in the parasitaemic compared to the aparasitaemic group of children, levels of haptoglobin (unlike CRP) levels are not correlated with parasite densities in infected individuals. This may imply that even a low degree of parasitaemia is sufficient to cause a significant reduction in plasma haptoglobin but could also be a reflection of the delay in return of haptoglobin levels to the normal range following a reduction in parasitaemia.

Bed net use was found to be associated with a decrease in the prevalence of hypohaptoglobinaemia, reflecting the effect of bed net use on malaria morbidity and confirming the finding of a previous study in The Gambia (Sisay *et al.*, 1992). Therefore, although the plasma haptoglobin level may not be useful in the diagnosis of disease episodes at the individual level, we found, as have others, that it may be useful as a surrogate index of endemicity at the community level (Trape *et al.*, 1985; Rougemont *et al.*, 1988; Hurt, 1994).

CRP and haptoglobin levels varied between the NIBP surveillance areas, reflecting different ecological and cultural differences in The Gambia. The proportion of children with an elevated CRP or low haptoglobin was significantly higher in the eastern part of the country than further west. This agrees with a baseline survey undertaken in 1991, in which the levels of malaria parasitaemia and splenomegaly were found to be highest in villages situated in the eastern areas 4 and 5 (Thomson *et al.*, 1994). Biochemical markers such as these could be developed as surrogate measures of relative malaria endemicity in different geographical regions. Since the measurement of CRP and haptoglobin by ELISA is simple and inexpensive, determination of their levels could also be considered as complementary or alternative means for the initial assessment of community -based intervention programmes, especially if cross-sectional, rather than longitudinal, surveillance is planned.

VII

Concluding Discussion

The host inflammatory response is a critical arm of innate immunity but may contribute to the pathogenic processes leading to severe malaria. In investigating the contribution of complex cytokine cascades to pathogenesis, the problem is to distinguish molecular mechanisms that are causal from those that are epiphenomena of the disease process. One of the strongest pieces of evidence for the pathological relevance of a putative molecular mechanism is to demonstrate that functional polymorphisms of the corresponding genes affect clinical outcome. The association of a genetic variant with the clinical outcome of disease strongly argues for the product of that gene, or of a closely linked gene, being involved in the pathological process (Lander & Schork, 1994). We have used this genetic approach to investigate the role of TNF in the development of severe malaria.

We have found the TNF promoter variant TNF_{-308} A (TNF2) to be associated with cerebral malaria in the Gambian case-control study of severe malaria and hypothesised that this association may be due to increased TNF production (Chapter II). Early evidence suggested that the TNF_{-308} A allele affected TNF production (Wilson *et al.*, 1994). Since this observation was made, several groups have further examined the functional aspects of the TNF_{-308} A allele at the cellular level. Increased TNF production is associated with TNF_{-308} A in some studies (Bouma *et al.*, 1996; Kroeger *et al.*, 1997; Wilson *et al.*, 1997), but not all (Brinkman *et al.*, 1996; Stuber *et al.*, 1996). A number of groups have described associations with the TNF_{-308} A allele with other infectious diseases. These include leishmaniasis (Carbera *et al.*, 1995), meningococcal septicaemia (Nadel *et al.*, 1996), trachoma (Conway *et al.*, 1997) and leprosy (Roy *et al.*, 1997).

With regard to the TNF_{-238} A allele, found to be associated with severe malarial anaemia (Chapter III), there are few data on its functional importance. Only one study has been published and this failed to demonstrate an effect on TNF response to short term stimulation with bacterial lipopolysaccharide (Pociot *et al.*, 1995). However, it is unclear whether TNF production following chronic stimulation of macrophages or monocytes by *P. falciparum* would be affected by the TNF_{-238} A allele; further studies are required.

We recognise that the disease associations that we have described may be due to the existence of other alleles in linkage disequilibrium with the TNF promoter alleles, either in

TNF or in a neighbouring gene. Although we have demonstrated both associations to be independent of variation in the HLA-class I and class II region, the MHC, including the HLA-class III region, may contain up to 200 immunologically relevant genes. Additionally, the functional importance of more recently defined point polymorphisms in the TNF promoter has yet to be investigated (Hamann *et al.*, 1995; Zimmerman *et al.*, 1996).

The importance of the associations that we have described in a Gambian population may be investigated by determining if the same associations also exist in a genetically diverse population (in East Africa, for example). However, undertaking further large case-control studies in rural Africa is logistically difficult. A major problem in many populations is ethnic admixture; a confounding variable for genetic association studies (Lander & Schork, 1994). Fortunately, the Gambian population consists of several very clearly defined ethnic sub-groups and population stratification could be accounted for when the associations were analysed. An alternative approach, which obviates the confounding effect of ethnic admixture, is family-based association studies where the parental alleles not inherited by the cases serve as the control 'group'. This strategy may be more applicable than traditional case-control studies to the future investigation of genetic associations with malaria in African children (Hill, 1997).

The genetic approach may be applied to the investigation of the putative role of several other host inflammatory mediators in the pathogenesis of malaria. We have discussed the difficulty of defining the pathogenic importance of the soluble TNF receptors and of the endothelial receptor, ICAM-1, by analysing plasma levels of the proteins (Chapters IV and V). Recently, Kurtzhals *et al.* (1998) have measured plasma levels of TNF and interleukin-10 in African children with malaria and suggested that severe malarial anaemia is related to an insufficient counter-inflammatory response (IL-10) to high TNF concentrations. Similarly, there is controversy regarding the role of nitric oxide in the pathogenesis of severe malaria; the currently available clinical data give conflicting findings based on measurement of plasma reactive nitrogen intermediates (Al Yaman *et al.*, 1996; Anstey *et al.*, 1996; Kremsner *et al.*, 1996). Supportive evidence for the involvement of these mediators in the pathogenesis of malaria may be sought via association studies using polymorphic markers in the genes coding for these molecules. For example, a recent study

in Kenya demonstrated an association of a high frequency polymorphism in the binding domain of ICAM-1 with susceptibility to cerebral malaria thus implicating this molecule in the pathogenic process (Fernandez-Reyes *et al.*, 1997). However, it should be stressed that although this approach has great potential, caution should be exercised in the interpretation of associations defined. In addition to the issue of population stratification in case-control studies discussed above, it is important to recognise that increased efficiency in molecular biology techniques could result in multiple-hypothesis testing, aggravated by publication bias of positive associations. Statistical correction for multiple testing is essential before commenting on the significance of genetic associations.

In addition to the candidate gene approach discussed above, advances in automated molecular techniques to characterise polymorphic microsatellite markers and computer software to construct high resolution genetic maps will allow genetic linkage studies covering the entire genome to be undertaken. Whereas the candidate gene approach requires a prior hypothesis for the involvement of a given mediator in pathogenesis, the whole genome approach can lead to the identification of new major susceptibility genes which may allow novel pathological pathways to be uncovered. Marquet *et al.* (1996) have used this approach to identify a gene in a region on the short arm of chromosome 5 that governs intensity of infection to *Schistosoma mansoni*. Intriguingly this region encodes IL-4, IL-5 and several other immunological mediators. In the case of malaria, it may be that in addition to those genes recognised to be of importance from association studies, other unidentified genes of greater importance are yet to found.

The aim of exploring the molecular mechanisms involved in a complex disease such as severe malaria is to improve therapeutic or preventative strategies. In the case of TNF, the trial of anti-TNF monoclonal antibody therapy in cerebral malaria in Gambian children resulted in a disappointing outcome (Boele van Hensbroek *et al.*, 1996). However, use of pentoxifylline, an inhibitor of TNF production, has been more promising and may merit further study (Di Perri *et al.*, 1995). With regard to prevention, several groups have endeavoured to define the parasite moieties that induce the host inflammatory response *in vivo* as these may form the basis of an 'anti-disease' malaria vaccine (Playfair *et al.*, 1990). Current evidence suggests that the TNF-inducing factors contain an inositol-containing

phospholipid, possibly glycosylphosphatidylinositol, but further molecular definition is required (Bate *et al.*, 1992; Bate & Kwiatkowski, 1994; Schofield & Hackett, 1993; Schofield *et al.*, 1996; Tachado *et al.*, 1997). In addition, before the case for an anti-disease vaccine can be advanced, an improved understanding of the molecular basis and the epidemiological significance of the natural anti-toxic antibody response in malaria-endemic populations is required (Kwiatkowski *et al.*, 1997).

In conclusion, the continued investigation of the role of the host inflammatory response in the complex pathogenesis of severe malaria in African children may ultimately lead to improvements in therapeutic or preventative strategies. This should continue in parallel with the on-going study of the many overlapping molecular processes involved in the course of malaria infection, such as the adhesion of parasitised erythrocytes to vascular endothelium (Newbold *et al.*, 1997). Crucially, such research should be regarded as complementary to the continued study of the epidemiology of severe malaria (Snow *et al.*, 1997) and to the development of 'low-technology' community-based interventions such as use of insecticide-impregnated bed nets (D'Alessandro *et al.*, 1995a). A coordinated, multi-disciplinary effort in the laboratory and in the field is essential if the devastating consequences of malaria infection on the population of sub-Saharan Africa are to be reduced.

VII

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VII

Acknowledgements

I thank Dominic Kwiatkowski for his inspiration, patience and support throughout and beyond the course of this work.

I owe very grateful thanks to many other individuals; most importantly to the Gambian children and families who consented to involvement in the various field and clinical studies.

I am extremely grateful to Adrian Hill and Richard Moxon for providing facilities, advice and encouragement and to Clive Bate, Ian Scragg, Nicola High and Mary Deadman for friendship and much help while at the Institute of Molecular Medicine, Oxford.

I am indebted to Umberto D'Alessandro, Brian Greenwood and Patricia Langerock and to medical, nursing and technical colleagues for their enthusiastic support during my time in The Gambia.

Finally, I gratefully acknowledge the support of The Wellcome Trust.