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Cerebral Malaria in Children: Serum and Cerebrospinal Fluid TNF-α and TGF-β Levels and Their Relationship to Clinical Outcome

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Summary

This was a prospective study conducted at the Moi Teaching and Referral Hospital, Eldoret, Kenya. Twenty-three children admitted to the hospital with cerebral (CM) and 10 children with non-cerebral malaria (NCM) were studied. The aim of the study was to establish and compare levels of tumour necrosis factor (TNF-α) and transforming growth factor (TGF-β1) in these children. Serum and cerebrospinal fluid (CSF) cytokine levels were assayed using ELISA kits. In serum, TGF-β1 and TNF-α decreased over 5 days after admission to the hospital in both groups of patients with CM and NCM. In the CSF of cerebral cases the levels of TNF-α and TGF-β1 were low and inversely related. Children in deeper coma had lower levels in serum of TGF-β and higher levels of TNF-α than those in lighter levels of coma. The serum TNF-α levels in CM children were the same irrespective of the duration of illness before admission, but children with NCM who had been sick for a shorter duration before admission tended to have higher serum levels of TNF-α and higher levels of TGF-β than those with a longer duration of illness before admission. In conclusion, this study shows that TNF-α and TGF-β1 may not be useful in predicting the outcome for CM. They may, however, be useful in detecting children at risk of developing deep coma. TNF-α and TGF-β levels were inversely related both in serum and CSF.

Introduction

There are an estimated 350 million cases of malaria in sub-Saharan Africa every year with over one million deaths, most of which are due to cerebral malaria (CM).[1,2] Earlier studies on the role of tumour necrosis factor-α (TNF-α) in the pathogenesis of malaria showed low serum levels at physiological concentrations to be beneficial while too high levels were harmful and associated with severe malaria, especially CM.[3–5] Furthermore, organ injury correlated with high circulating TNF-α and IL-6 levels.[6] In 65 Malawian children with severe malaria, mortality was observed to increase with increasing serum TNF-α levels[7] and in Gambian children plasma TNF-α levels were found to be higher among those with CM than in those with uncomplicated malaria (UM).[8] However, there are conflicting results, including some studies showing that plasma TNF-α levels were high in all forms of severe malaria except in CM[9] and others reporting high plasma TNF-α levels in children with UM and not in CM.[10,11] Therefore, serum TNF-α levels may not be used as the only indicator of malaria severity.[11] Interestingly, McGuire, *et al*.[12] found that there is a genetic predisposition to CM and that high levels of TNF-α implies a higher risk of getting and dying from CM. Associations of TNF-α promotor single-nucleotide polymorphisms with susceptibility for, or protection from, severe malaria have also been reported.[13,14]

Malaria parasites in the blood are thought to initiate intermittent TNF-α production by macrophages, which is responsible for the rupture of schizonts and the release of parasites into circulation with resultant pyrexia.[15,16] The effect of TNF-α has been tested by the administration of monoclonal anti-TNF-α antibodies resulting in suppression of fever in children with severe malaria.[17] High serum TNF-α levels were also associated with high fever, parasitaemia, and hypoglycaemia through inhibition of

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TABLE 1
Patient characteristics

	Cerebral malaria $n = 23$			Uncomplicated malaria $n = 10$		
	Mean	Median	Range	Mean	Median	Range
Duration of illness (days)	7	3	1–8	5	3	1–7
Temperature on admission day 1 (°C)	40.8	38	35–41	39	37	36–41
Parasite count on admission per microlitre	223085	36936	924–1862656	506890	54272	1234–945671
Haemoglobin on admission (g/dl)	10.5	10.8	6–14.9	9.5	10.3	6–12.6
Blood sugar on admission (mmol/l)	7.1	7.4	2–12.9	5.8	4.5	2–9.8
Age (years)	6	6	2–11	5	4	1–12

gluconeogenesis in cases of severe malaria.[6,7,17–20] However, our own studies on fever showed no statistical difference between CM and UM in brain, core, and skin temperatures.[21,22] Few studies have focused on TNF-α levels in the cerebrospinal fluid (CSF). Some have shown high CSF TNF-α levels in children dying of CM, while others found lower TNF-α levels in CSF than in serum in severe malaria.[2,7,23]

Transforming growth factor β (TGF-β) is thought to protect against severe pathology in murine malaria by controlling parasite growth; however, it is not clear whether this is the case in humans.[24] TGF-β is thought to be a natural antagonist to TNF-α as demonstrated in mice infected with malaria by a decrease in serum TNF-α on administration of recombinant TGF-β.[25] Further evidence of this antagonism was demonstrated by low serum TGF-β levels in mice with CM, and increased TNF-α mRNA levels and decreased TGF-β levels in the brain tissue of mice with CM.[25,26] A corresponding situation in humans was suggested by a study in Thailand in which patients with malaria revealed serum TNF-α levels inversely related to serum TGF-β levels.[27] Low levels of serum TGF-β were noted to increase after treatment with artesunate and mefloquine, but no correlation to parasitaemia was found.[27] Furthermore, serum TGF-β levels were found to be inversely related to the severity of malaria infection, i.e. lethal infections were associated with low levels and mild infections with high serum TGF-β levels.[2,24]

Although TGF-β has a crucial role in inflammation and repair,[28] the role of TGF-β in CM is not known.

The aim of the present study was to estimate serum levels of TNF-α and TGF-β in children with CM and non-cerebral malaria (NCM), their CSF levels in CM cases, and establish their roles in the pathogenesis of CM.

Materials and Methods

Patients and study population

The study included 23 children with CM and 10 children with NCM of which seven cases had UM, two had hyperparasitaemia, and one had hypoglycaemia. These were patients admitted to the paediatric wards of Moi Teaching and Referral Hospital, Eldoret and were consecutively recruited into the study over the study period May–September 1997 (Table 1). The number of patients in the study decreased because as they got better some of them absconded before completing the 5 days of the study. In this study, therefore, there were 23 CM and 10 NCM cases on admission (day 1), 21 CM and nine NCM on day 3, and 13 CM and four NCM cases on day 5.

The children were aged between 1 and 12 years (mean age 6 years). There were 11 females and 22 males. The study was conducted in the highlands of western Kenya in Eldoret where malaria occurs in epidemics as the inhabitants are non-immunes, unlike those in the endemic lowlands that have developed some immunity due to chronic exposure to the parasite. Children were considered to have malaria when they had detectable asexual forms of *Plasmodium falciparum* in the peripheral blood smear.

CM was diagnosed in those with unarousable coma after assessment using the Blantyre coma scale.[29] Coma grading was done by two independent clinicians on admission and then twice daily until a coma scale of 4 was obtained on two consecutive occasions. In cases where the two clinicians disagreed in their score, a third clinician was requested to carry out an independent score and the two of the three that agreed was considered the final score. Scores of 1–3 were considered coma, with 1 being the deepest level of coma.

Children with fever who had positive blood slides but who were not in coma, with no other features of complicated malaria, were considered to have UM. Children with parasite densities of more than 100 000/ml were classified as hyperparasitaemia, those with serum blood sugar less than 2.2 mmol/l were classified as hypoglycaemia, and those with haemoglobin levels less than 5 g/dl were classified as severe anaemia. These three latter categories were included together with the UM cases in one group,

i.e. 'non-cerebral malaria' (NCM), for the purposes of this study.

Parasite counts and haemogram were done in all patients on admission (day 1), day 3 and day 5 for both CM and NCM. Temperature was monitored every 6 h in both groups using the zero heat flow thermometer.[22] A lumbar puncture was done in all cases of CM to exclude meningitis. Patients with meningitis, head injury or mixed malaria and meningitis were excluded from this study.

Ethical considerations

Approval was obtained from the Research and Ethics committees of the Faculties of Health Sciences in Moi and Linköping Universities. Informed and written consent was obtained from the parents or guardians of all children before inclusion into the study.

Treatment regimens

Patients admitted into the study were asked if they had been on antimalarials before coming to the hospital and those who had been on antimalarials within 72 h before admission were excluded from the study.

Patients with CM were then treated with intravenous quinine 20 mg/kg in 500 ml of 5 per cent dextrose run over 4 h. This was followed by two doses of 10 mg/kg in 500 ml of 5 per cent dextrose run over 16–20 h, and thereafter this dose was repeated every 8 h until the patient was out of coma when they were changed to intramuscular or oral quinine. This treatment regimen was also used for NCM cases for the first 24 h and was then changed to oral or intramuscular quinine until the 5th day. After the 5th day all patients were discharged on oral quinine for 3–5 days for both groups of cases.

Methods

A lumbar puncture was performed at admission on all children with a suspicion of CM, and bacterial meningitis was excluded through bacteriological and biochemical analysis of CSF. Five millilitres of peripheral blood was drawn on admission (day 1), days 3 and 5 for analyses of haemogram, blood sugar, malaria parasites and cytokines. For all children on admission (day 1) samples were drawn before any antimalarial or supportive treatment was commenced. Samples for cytokines were centrifuged within 15 min after collection and immediately frozen at –20°C and were subsequently (within 24 h) transferred to the –70°C freezer. The samples were later thawed at room temperature and TNF-α and TGF-β were analysed using commercial ELISA kits at the Immunology Department of Moi University.

ELISA kits for TNF-α and TGF-β were obtained from Biosource S.A., Belgium. These are high quality kits, each including two internal controls in addition to the standards. The ELISA tests were performed according to the manufacturer's instructions. In order to compensate for the different matrixes in serum and CSF, the CSF was mixed with 'Special point zero' (Biosource), and then further treated as the sera. 'Special point zero' consists of human plasma that has been screened for cytokines and supplied with benzamidin 1g/l, thymol 0.2 g/l and ethanol 2 ml/1. All samples were run in duplicate and the mean values were used.

Statistical methods

Non-parametric tests were used in the analysis due to the small sample sizes in the study. The Mann–Whitney was used for comparisons between groups and the Spearman's rank correlation test was used to compare the different parameters.

Results

The two groups of patients, CM and NCM, had comparable clinical and laboratory parameters ($p \geq 0.05$) during recruitment as depicted in Table 1.

Serum levels of TNF-α and TGF-β

Serum levels of TNF-α were high on admission in both CM and NCM cases and decreased with time while on treatment, with the lowest levels reported on day 5 (Fig. 1a). There was no statistical difference between the CM and NCM cases.

Serum levels of TGF-β1 showed a similar pattern with high levels on admission, decreasing with time (Fig. 1b). No statistical difference between the two groups was observed.

There was a negative correlation between serum TNF-α and TGF-β1 levels in both groups with correlation coefficients of $r = –0.528$ on day 1 ($p = \leq 0.05$) and $r = –0.540$ on day 3 ($p = \leq 0.05$). When the TNF-α levels were high, the TGF-β1 levels were low and vice versa in CM patients (Fig. 2a, b).

CSF levels of TNF-α and TGF-β

The levels of TNF-α and TGF-β1 in CSF (Fig. 3a, b) were generally low on admission (day 1). Of the 23 cases with CM, 17 had no detectable cytokine levels in their CSF. It was observed that when TNF-α levels were measurable, detectable TGF-β1 levels were very low and vice versa. The range for TNF-α levels was 0–18 pg (median 0, mean 1.6 pg) and for TGF-β the range was 0–2 pg (median 0, mean = 0.095 pg). There was also a weak positive correlation between serum and CSF TNF-α levels ($r = 0.508$, $p \leq 0.05$) and a very weak negative correlation between serum and CSF TGF-β levels ($r = –0.366$, $p \leq 0.05$) in these patients (data not shown).

Parasitaemia

There was a positive correlation between parasitaemia and serum TNF-α levels for both CM and

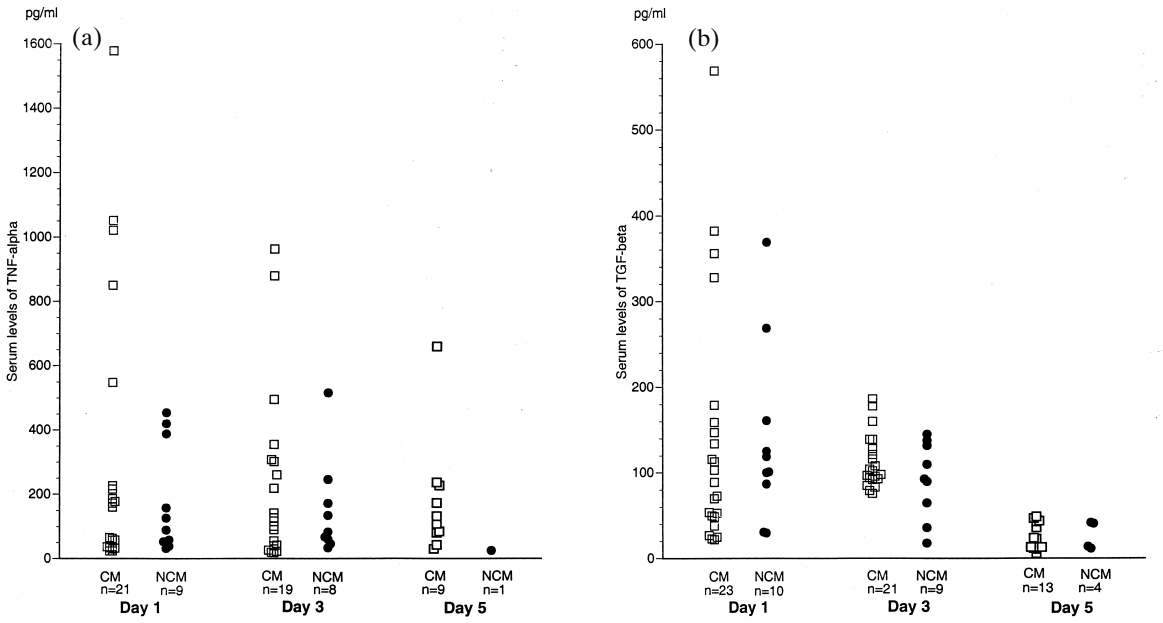

FIG. 1. (a) Serum TNF-α levels for cerebral (CM) and non-cerebral (NCM) malaria on days 1, 3 and 5. (b) Serum TGF-β for CM and NCM malaria on days 1, 3 and 5.

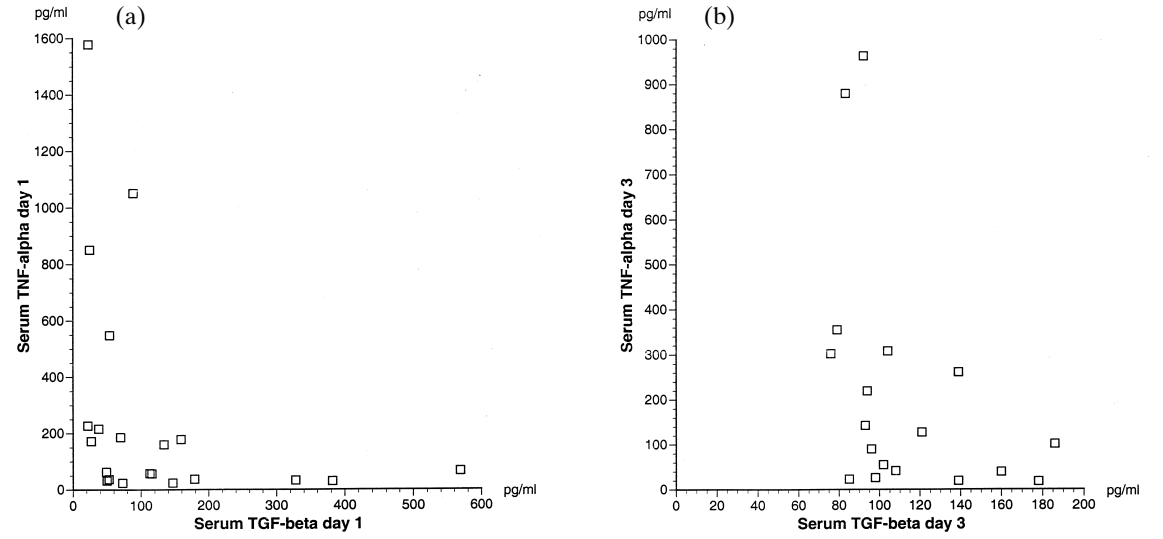

FIG. 2. (a) Correlation between serum TNF-α and TGF-β on admission (day 1) for cerebral malaria (CM) patients. (b) Correlation between serum TNF-α and TGF-β on day 3 for CM patients.

NCM cases ($r = 0.596$ and $r - 0.3$, respectively; $p < 0.05$), but not for TGF-β (data not shown).

Haemoglobin levels

There was no significant correlation between haemoglobin levels and serum TNF-α or TGF-β1 levels in both CM and NCM cases (data not shown).

Body temperature

There was no clear correlation between body temperature and serum TNF-α or TGF-β1 on days 1, 3 and 5 for CM and NCM cases (data not shown).

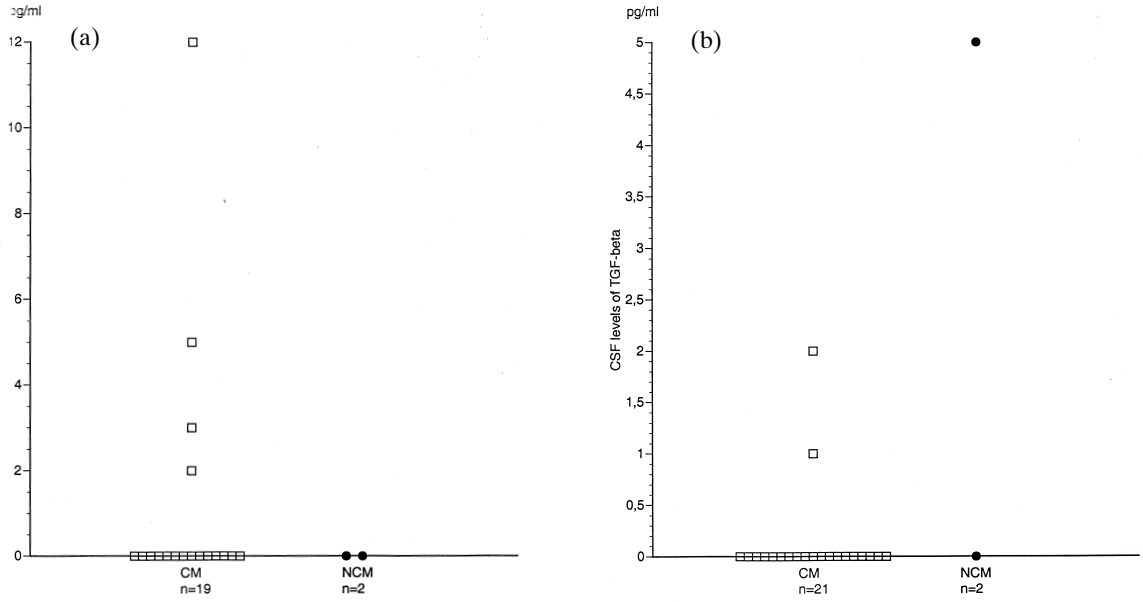

FIG. 3. (a) CSF levels of TNF-α in cerebral malaria (CM) patients on admission. (b) CSF levels of TGF-β in CM patients on admission.

Duration of illness

The serum TNF-α levels were the same irrespective of duration of illness in CM. In children with NCM, TNF-α levels in serum tended to be higher for those with a shorter duration although this was not statistically significant (Fig. 4a).

The serum TGF-β1 levels tended to be higher for children with shorter duration and lower for those with longer duration of illness before admission in both CM and NCM cases, however this was not statistically significant (Fig. 4b).

Coma scale on admission

There were higher levels of TNF-α in children with deeper coma than those with lighter degrees of coma and the opposite for TGF-β levels (Fig. 5a, b). This was statistically significant ($p = 0.03$ and $p = 0.04$, respectively).

Discussion

Cytokines are known to be important mediators in the pathogenesis of human malaria. TNF-α has been found to be both beneficial and harmful depending on serum levels. High levels are associated with severe disease although some patients with very high TNF-α levels may have less severe malaria. Low serum levels are usually associated with mild illness.[10–12] We found no statistical difference between the serum TNF-α levels in CM and NCM cases which supports previous studies that show high TNF-α to be associated with severe malaria, but not specifically CM.[7,9,19,20]

The TNF-α production may be dependent on the *P. falciparum* strain responsible for the malarial infection.[30] There is, however, no evidence that high TNF-α inducing strains are associated with CM, although Allan *et al.*[30] found high TNF-α inducing strains among CM cases when compared with those causing NSM. There was, however, considerable overlap in the TNF-α levels between the CM and NCM cases.

This inconsistency in results could be due to the dual role TNF-α plays in malarial infection, i.e. excessive production causes illness, but is also associated with protective immune responses.[31] T helper type (Th1) like responses are needed for clearance of the intracellular malarial infection,[31–33] and CD8+ T cell responses seem to be of particular importance.[34] This seems to support our findings and provides the possible explanation for why the levels of TNF-α may not be a good indicator for CM.[7,9,19,20]

We found higher serum TNF-α levels among children who were in deeper coma than those in lighter degrees of coma on admission. In contrast, the serum TGF-β1 levels were lower in children in deep coma on admission, showing an inverse relationship with serum TNF-α levels. There are no previous studies that relate coma and serum TNF-α or TGF-β1 levels and therefore further studies are needed to corroborate our findings. Furthermore, these was a positive correlation between TNF-α

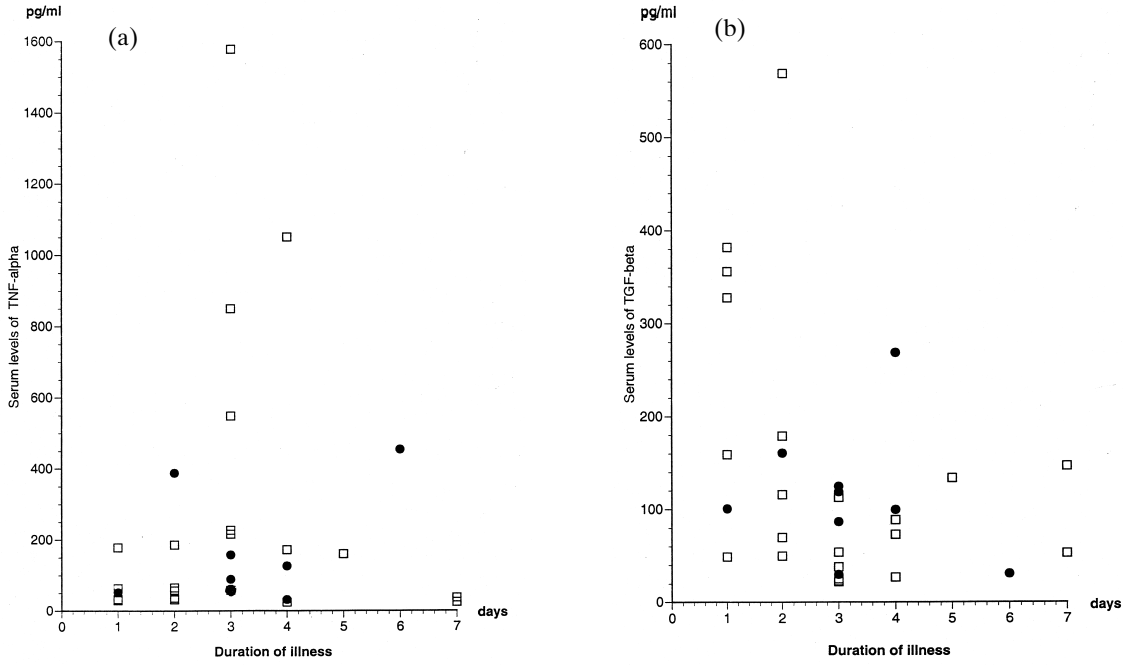

FIG. 4. (a) Relationship between serum TNF-α levels and duration of illness before admission: □, cerebral malaria (CM) $n = 21$; ●, non-cerebral malaria (NCM) $n = 9$. (b) Relationship between serum TGF-β levels and duration of illness before admission: □, CM $n = 23$; ●, NCM $n = 10$

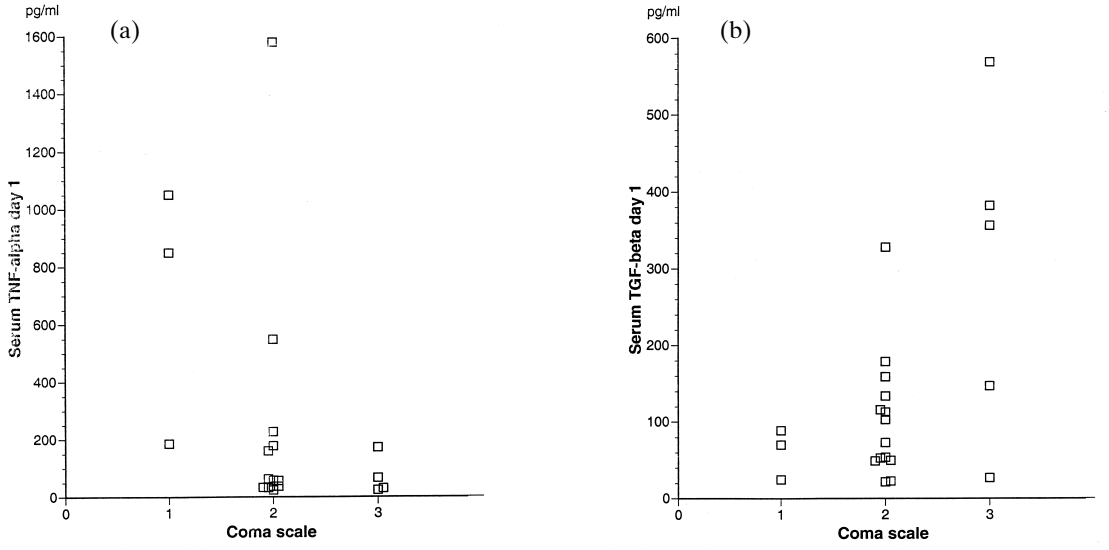

FIG. 5. (a) Relationship between serum TNF-α levels and level of coma (as described in Materials and Methods) in cerebral malaria (CM) patients on admission (day 1), $n = 21$. (b) Relationship between serum TGF-β levels and level of coma in CM patients on admission (day), $n = 23$.

levels in serum and parasitaemia levels in both CM and NCM patients on admission but not for TGF-β1 levels, which corroborates previous reports.[7,8,18,19,27]

We observed that the serum TGF-β1 levels were similar in CM and NCM cases, which also agrees with studies suggesting that TGF-β1 is not specific to cases with CM.[35]

TGF-β is an overall anti-inflammatory cytokine, which exerts regulatory effects on macrophage function including suppression of IFN-γ induced activation.[36] It also enhances IL-10 production,[37] which has been reported to be protective and inhibitory to the production of TNF-α in experimental CM.[38] Moreover, decreased IL-10: TNF-α ratios were recently shown to be a risk factor for CM and severe anemia.[39,40] We could not however, find any differences in TGF-β: TNF-α plasma level ratios between NCM and CM. TGF-β may be a dampener of the immune response in malarial disease, protecting the host from immune-mediated side-effects, or it may turn off the immune response at a too early stage, before the infection is adequately eradicated.[41]

The CSF TNF-α and TGF-β levels among the CM cases were in this study mostly undetectable and when present they were generally very low. Brown, *et al*.[35] looked at cytokine expression in brains of children dying of CM, meningitis, and encephalitis at post mortem and found high levels of TNF-α and low levels of TGF-β1 in all these conditions, suggesting that they are not specific for CM.[35] Our findings of low levels of TNF-α and TGF-β in CSF are not due to methodological errors since in a study on neuroborreliosis, using the same methods, we found high levels of TGF-β in the CSF (Widhe MEA, *et al*., unpublished). However, the presence of TNF-α and/or TGF-β1 in CSF could not be caused by a disturbed blood–brain barrier, because in CM the blood–brain barrier is intact,[41,42] although this has been questioned in a recent study in which subtle changes were reported.[43]

The observed inverse relationship between TNF-α and TGF-β1 both in serum and CSF, especially on admission, is consistent with earlier findings.[26,27] When TNF-α levels were high, the TGF-β1 levels were low and vice versa, which is in support of the theory that they are functionally antagonistic.[2]

There was a positive correlation between serum and CSF levels of TNF-α. Thus children with high serum levels of TNF-α also had high CSF levels of TNF-α which agrees with previous findings.[20] Regarding TGF-β1, a negative correlation between serum and CSF levels was demonstrated, which has not been reported previously.

Studies on cytokines seem to show contrasting results which may be related to temperatures at which the samples were stored since cytokines degrade faster at higher temperatures. Differences have also been observed when the same sample is assayed by different methods. The ELISA methods were found to have the highest sensitivity when compared with radioimmunoassay (RIA) and bioassay methods. RIA, however, has been found to yield higher levels.[27,44] The ELISA method was used in our study.[45]

Conclusions

No differences were observed between CM and NCM cases with regard to serum levels of either TNF-α or TGF-β. In the CSF of CM cases the levels of TNF-α and TGF-β1 were low and inversely related. Thus, this study shows that levels of TNF-α and TGF-β1 may not be used as an indicator for CM but may be useful as an indicator of the risk of developing deep coma. The study also confirms the antagonistic actions of TNF-α and TGF-β1.

The findings in this study should be corroborated with a larger study as they reveal some important aspects that relate to cytokines and malaria infection.

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