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Elevated levels of soluble CD14 in serum of patients with acute *Plasmodium falciparum* malaria

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SUMMARY

Serum sCD14, tumour necrosis factor-alpha (TNF-α), IL-6, and endotoxin were analysed in 45 patients with complicated malaria, in 14 patients with Gram-negative septicaemia and in 24 healthy subjects by ELISA. Malaria patients with renal failure (n=16) had higher levels than patients without renal failure (n=29) (8116+1440 μg/*l versus* 9453+1017 μg/*l*; P<0·05) and both had higher levels than patients with septicaemia (6155+1635μg/*l*) and normal subjects (2776+747 μg/*l*). A significant correlation between sCD14 and IL-6 (r=0·756) and TNF (r=0·822) existed. However, no relation between sCD14 and serum endotoxin or indices of clinical disease severity (parasitaemia, fever, parasite or fever clearance time) was seen. Although the role of sCD14 in malaria remains to be determined, elevated levels may participate in the inflammatory response in complicated malaria.

Keywords *Plasmodium falciparum* malaria soluble CD14 septicaemia tumour necrosis factor

INTRODUCTION

CD14 as a 53-kD phosphatidylinositol-linked membrane glycoprotein is a marker of human monocytes and serves a receptor for endotoxin [1,2]. A soluble form of CD14 (48-kD sCD14) has been demonstrated in supernatants of cultured $CD14^+$ cells [3] as well as in serum and urine [4,5]. Although the serum concentration of sCD14 is $\sim$ 1 million-fold higher than that of cytokines [6], its physiological role is unknown. It could act as a scavenger by neutralizing circulating lipopolysaccharide (LPS) [17] or, alternatively, it may play a harmful role by transmitting LPS effects onto endothelial cells [8]. In *Plasmodium falciparum* malaria elevated serum concentrations of endotoxin-like parasite antigens and cytokines (including tumour necrosis factor (TNF), IL-6, IL-8) have been described [9–11].

The interaction between CD14 and endotoxin was shown to liberate TNF-α, which has been implicated in the pathophysiology of complicated malaria [9]. We measured serum concentrations of sCD14, endotoxin, TNF-α, and IL-6 in 45 patients with complicated *P. falciparum* malaria (and active exclusion of individuals with secondary bacterial infection), and compared the values with concentrations in patients with Gram-negative septicaemia (n = 14) and healthy subjects (n = 24).

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PATIENTS AND METHODS

Study populations

Patients with severe *P. falciparum* malaria conforming to the following criteria were included in the study: (i) severe malaria: (ia) unarousable coma; (ib) renal impairment (urine output <400 ml/24 h in adults (12 ml/kg per 24 h in children) and a serum creatinine >3·0 mg/dl); (ic) pulmonary oedema or acute respiratory distress syndrome (tachypnoea and exclusion of bronchopneumonia and/or acidosis by clinical findings and chest x-ray); (id) severe normocytic anaemia (packed cell volume <15% (haemoglobin <5 g/dl); (ie) hypoglycaemia (blood sugar <40 mg/dl (2·2 mmol/*l*)); (if) hyperlactataemia (lactate levels >6 mmol/*l*); (ig) hyperpyrexia (rectal temperature >40°C); (ih) hyperparasitaemia (>2% parasitaemia in non-immune); (ii) jaundice (serum bilirubin >3·0 mg/dl); (ij) circulatory collapse or shock (systolic blood pressure <70 mmHg with cold clammy skin or core-skin temperature difference >10°C; (ik) spontaneous bleeding (bleeding from the gums, nose, GI tract, etc., and/or DIC); (il) repeated generalized convulsions (more than two observed within 24 h); (im) acidaemia or acidosis (arterial pH <7·25 or plasma bicarbonate <15 mmol/*l*); (in) macroscopic haemoglobinuria; (ii) patient's age more than 12 years; (iii) weight 30–80 kg; (iv) either patient or family member willing to give written informed consent; and (v) *P. falciparum* parasitaemia.

Reasons for exclusion were: (i) concurrent major illness (diabetes mellitus, acute and chronic heart failure, etc.); (ii)

pregnancy (if female, the patient must be surgically sterilized, post-menopausal, or have a negative pregnancy test); and (iii) CSF examination; protein >200 mg/dl; Gram stain positive for micro-organism; total cell count >100/mm^3; other findings of viral encephalitis, bacterial meningitis or trauma.

A general physical examination was performed on admission to the study. Clinical efficacy was assessed by recording the duration of coma (cerebral malaria), duration of respirator therapy (respiratory failure), parasite clearance, fever, duration of dialysis (renal impairment), acidosis, incidence of other complications associated with severe malaria.

This study was approved by the Ethical Committee Board of the Mahidol University. Written informed consent for each patient was obtained in accordance with Title 21, Parts 50 and 56 of the US Code of Federal Regulations.

Antimalarial treatment

All patients received standard antimalarial treatment with artesunate (120 mg statim i.v., 60 mg every 12 h to a total of 600 mg). The response to antimalarial therapy was measured according to the standardized classification system designed by WHO (Technical Report Series 529, p.30). The rates of parasite clearance from peripheral blood and disappearance of fever were considered corroborative evidence of efficacy. Parasite clearance times were calculated from initiation of treatment until the first time that peripheral blood films were negative for asexual parasites. Fever clearance times were calculated from initiation of treatment until the first time that the temperature decreased 37°C, and remained less than 37°C for at least 24 h.

Laboratory assessments

For routine patient care, parasite counts were examined four times daily (at 6-h intervals) until blood films were negative on three consecutive examinations by Giemsa-stained thick smears. Weekly smears were performed on days 7, 14, 21 and 28. Routine urinalysis and haematological tests were performed before treatment, every 24 h for 7 days after initiation of treatment, and on study days 14, 21 and 28.

Blood samples were obtained before and after initiation of treatment on days 1, 7, and 21. The blood specimens were centrifuged at 4°C (900 g/15 min), serum separated and immediately frozen at −70°C. Serum concentrations of sCD14 were determined by sandwich ELISA (Immuno Biological Laboratories, Hamburg, Germany). A polyclonal anti-sCD14 was coated onto a microtitre plate. Circulating sCD14 was detected with a second biotin-conjugated MoAb. In normal controls the mean serum level of sCD14 was 2776 + 747 μg/*l*. Serum concentrations of endotoxin were determined with the limulus amoebocyte lystate assay according to the instructions of the manufacturer (Coatest Endotoxin; Endosafe Inc., Charleston, SC) [12]. This assay is routinely used for patients in the intensive care unit of our hospital. The interassay and intra-assay coefficients of variation were <10% and <5% respectively. Normal values were <12·5 ng/*l*. Serum levels of IL-6 and TNF-α were determined by ELISA (Biomedica, Vienna, Austria) as described [13,14]. Normal values were <13 ng/*l* and <6 ng/*l*, respectively.

Septicaemia and controls

Fourteen patients (six female and eight male) ranging in age from 16 to 65 years were investigated. All patients fulfilled the previously published criteria for sepsis syndrome, i.e. clinical signs of infection, fever (>38·3°C) or hypothermia (<35·6°C), tachycardia (>90 beats/min), tachypnoea (> 20 breaths/min while breathing spontaneously), and at least one of the following manifestations of inadequate organ perfusion or dysfunction: deterioration from baseline mentation, hypoxaemia (PaO^2 < 75 mmHg on room air in the absence of pulmonary disease), elevated lactate levels, oliguria (<30 ml/h or 0·5 ml/kg per h) (Bone RC). The pathogens isolated in multiple blood cultures before antimicrobial therapy were *Escherichia coli* (*n* = 10), *Salmonella typhi*, *Morganella morganii*, *Hafnia alvei* and *Klebsiella pneumoniae*. After obtaining the blood and urine cultures patients were treated empirically with cephalosporines. Patients were hospitalized for 14–21 days. After discharge patients were followed at the out-patient clinic. Twenty-four healthy subjects (hospital staff) were the controls. In all cases patients or the relatives gave informed consent.

Statistical analysis

Non-parametric tests were used. For comparison between patients and controls Kruskall–Wallis and Mann–Whitney–Wilcoxon *U*-test were used. Serum levels at different days were compared with the Wilcoxon rank sum test. For correlation analysis Spearman's test was used. All the analyses were two-sided, and differences with a *P* value <0·05 were considered statistically significant.

RESULTS

Clinical findings

Malaria. All patients (*n* = 45) were suffering from acute complicated falciparum malaria with a mean temperature on admission of 38·9± 0·6°C. Baseline data are depicted in Table 1. All patients had negative blood cultures. The most frequent symptoms were fever, headache, nausea, and backache. The mean parasite clearance time was 75 ± 17 h. The mean parasite counts before treatment were 226·740/μl. Fever persisted on average for 120 h after the start of treatment. Of 45 patients, 21 had coma, 16 of 45 had renal failure and eight patients had coma plus renal failure.

Septicaemia. Clinical data of septicaemia patients are displayed in Table 3. The most common diagnosis was urosepsis (eight of 14 patients). Underlying diseases were diabetes mellitus, renal stones, prostate hyperplasia, liver cirrhosis, chronic arterial occlusive disease with vascular prosthesis, and ileus. Twelve patients were clinically and bacteriologically cured.

One patient improved clinically and blood cultures became negative after 3 days of treatment. Due to persistence of periprosthetic abscesses, surgical therapy was necessary. One patient died

Table 1. Characteristics of patients with *Plasmodium falciparum* infection

Parameter	Mean (s.d.)
Age (years)	28 (12)
Sex (male/female)	25/20
Days with fever prior to treatment	4 (2)
Previous malaria attacks (number)	0·4 (0·2)
Highest temperature prior to therapy (°C)	38·4 (0·9)
Fever clearance time (h)	120 (89)
Parasite clearance time (h)	75 (17)
Initial parasitaemia (per μl)	226·740 (126·345)

Table 2. Serum levels of tumour necrosis factor (TNF) and IL-6 in complicated malaria (means ± s.d.)

Parameter	Day 0	Day 1	Day 7	Day 21	Normal values
IL-6 (ng/*l*)	289 ± 121	220 ± 101	14 ± 6	<13	<13
TNF (ng/*l*)	156 ± 113	86 ± 53	16 ± 4	<15	<15

due to multiorgan failure 4 days after initiation of treatment. The latter two patients were excluded from follow up.

Fever subsided on average after 7 days of therapy. Leukocytosis and serum levels of C-reactive protein were within normal range after 14 days of treatment, whereas blood sedimentation rate and serum levels of fibrinogen were within normal range 28 days after initiation of therapy. No steroids were used.

Serum concentrations of sCD14, TNF, IL-6 and endotoxin

Prior to therapy, elevated serum concentrations of sCD14 were found in patients with malaria ($8679 + 1241\ \mu g/l$) and in patients with Gram-negative septicaemia ($6155 + 1635\ \mu g/l$) compared with normal subjects ($2776 + 747\ \mu g/l$) (Fig. 1). In patients with both malaria and septicaemia serum sCD14 dropped in a delayed manner after initiation of therapy. Malaria patients with renal failure ($n = 16$) had significantly higher levels than patients without renal failure ($n = 29$) ($9453 + 1017\ \mu g/l$ *versus* $8116 + 1440\ \mu g/l$ before therapy (Fig. 1)). No correlation between sCD14 and serum creatinine or BUN ($P > 0{\cdot}05$ for both) was seen.

None of the controls and only two malaria patients had a positive assay for endotoxin, whereas all samples of patients with septicaemia were endotoxin-positive. Serum concentrations of TNF and IL-6 are shown in Table 2. High levels of both cytokines were seen before therapy, which normalized on day 7. A significant correlation between serum levels of sCD14 and serum levels of both TNF ($r = 0{\cdot}822$; $P < 0{\cdot}001$), and IL-6 ($r = 0{\cdot}756$; $P < 0{\cdot}001$) was seen. No relation between serum sCD14 and endotoxin or indices of clinical disease severity like parasitaemia or fever was seen. In addition, levels of sCD14 were not related to fever and/or parasite clearance time and/or to other organ manifestations like coma or respiratory failure.

DISCUSSION

In this study patients with complicated *P. falciparum* malaria had significantly elevated concentrations of sCD14 compared with patients with Gram-negative septicaemia and healthy subjects. In malaria patients with acute renal failure sCD14 levels were significantly higher than in patients with normal renal function. A significant correlation between sCD14 and IL-6 and TNF, but no correlation to endotoxin existed. Apart from renal failure no relation with clinical indices of disease severity and/or other organ manifestations was seen [15].

Soluble CD14 is spontaneously released by the Mono-Mac 6 cell line, by monocytes, macrophages, and granulocytes [16–19]. After release, sCD14 circulates in the bloodstream [6]. An increase in sCD14 in serum of patients with Gram-negative septicaemia [15] and patients with malaria could be due to a reduced clearance, or alternatively to enhanced liberation.

The stimulus for sCD14 may be endotoxin or triggering by cytokines. In parasite extracts no significant amounts of endotoxin were found [20]. Some authors found no circulating endotoxin in the serum of malaria patients [21,22]. Others found endotoxin in the plasma of malaria patients, and suggested that it derived either from the parasites themselves or from the patient's gut [11]. However, in this study [11] endotoxinaemia was also thought to reflect 'physiological disturbances' as in the cases of shock or stress, Gram-positive bacterial infection, or in liver diseases with associated renal failure, even without Gram-negative infection'. In the current study only two of 45 malaria patients (none had evidence of bacterial infection), but all 14 patients with Gram-negative septicaemia were positive for endotoxin. Since all controls were endotoxin-negative, the reason for the discrepancy between our and the previous study [11] regarding the number of endotoxin-positive samples could be related to occult bacteraemia in malaria [23,24] and/or contamination. Altogether, high levels of inflammatory cytokines and the resultant immune cell stimulation are probably implicated in sCD14 production [9,10,25,26]. In the

Table 3. Baseline data of patients with Gram-negative septicaemia

Patient no.	Age	Sex	Underlying condition	Causative pathogen	Diagnosis	Outcome	Comment
1	46	M	None	*Escherichia coli*	Urosepsis	Cure	
2	23	F	None	*E. coli*	Urosepsis	Cure	
3	63	M	Diabetes mellitus	*E. coli*	Urosepsis	Died	Multiorgan failure
4	19	F	Agammaglobinaemia	*Klebsiella pneumoniae*	Pneumonia	Cure	
5	31	M	None	*Salmonella typhi*	Typhoid fever	Cure	
6	16	F	None	*E. coli*	Septicaemia	Cure	
7	28	F	Renal stone	*E. coli*	Urosepsis	Cure	
8	65	M	Prostate hyperplasia	*E. coli*	Urosepsis	Cure	Renal insufficiency
9	25	F	None	*E. coli*	Urosepsis	Cure	
10	19	F	None	*E. coli*	Urosepsis	Cure	
11	54	M	Liver cirrhosis, psoriasis	*E. coli*	Septicaemia	Cure	Chronic alcoholism
12	34	F	None	*E. coli*	Urosepsis	Cure	
13	39	M	Abdominal trauma	*Morganella morganii*	Peritonitis	Cure	
14	65	M	Chronic arterial occlusive disease	*Hafnia alvei*	Vascular prosthesis Septicaemia	Improved	Persistence of periprosthetic abscesses

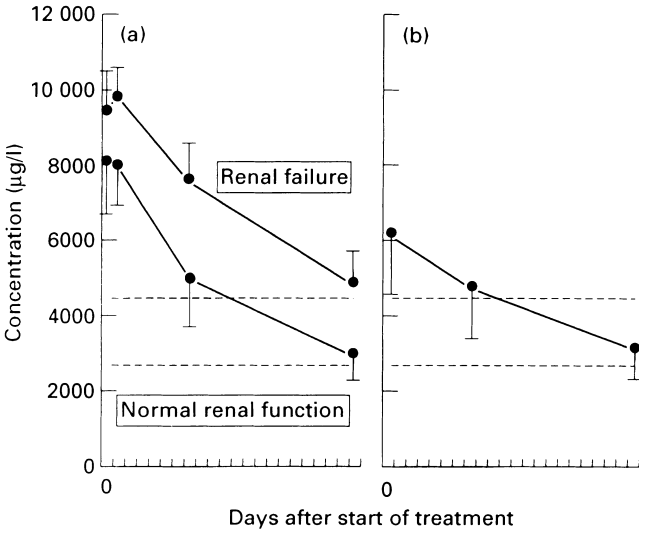

Fig. 1. Mean (± s.d.) serum concentrations of sCD14 in patients with *Plasmodium falciparum* malaria with and without renal failure (a) and in patients with Gram-negative septicaemia (b). The dotted lines denote normal range.

individual host, convalescence of bacterial infections or concomitant infections with LPS-producing organisms may vary the parasitaemia required to produce illness [27]. In this respect, LPS components have been used as malaria vaccine adjuvants to enhance the vaccine-directed immune response [28]. On the other hand the elevation of sCD14 in severe malaria could explain the impaired endotoxin detoxification as a factor in enhanced endotoxin sensitivity in malaria-infected mice [29].

sCD14 could promote (in concert with inflammatory cytokines) endothelial cell activation and thus adhesion molecule expression [30,31]. These adhesions molecules (e.g. intercellular adhesion molecule-1, endothelial leucocyte adhesion molecule-1, and vascular cell adhesion molecule-1) are receptors for erythrocytes infected with *P. falciparum* [32–34]. The adherence of infected erythrocytes to post-capillary endothelium in peripheral tissues avoids their clearance in the spleen or liver and contributes to evasion of the host immune response, as well as to the pathogenesis of severe malaria [35].

Soluble CD14 is closely related to TNF, which has been implicated in malaria pathology. This corresponds to several findings. Monocytes and neutrophils respond via CD14 by releasing TNF [36]. Second, superoxide radicals have been implicated in malaria-related tissue damage [25] and CD14 antibodies have been demonstrated to inhibit priming of polymorphonuclear leucocytes for enhanced release of superoxide [37]. *In vitro* pentoxifylline, a methylxanthine which decreases TNF release via the inhibition of phosphodiesterase and the increase of intracellular cyclic adenosine monophosphate [38], and CD14 antibody additively inhibited priming of polymorphonuclear leucocytes for enhanced release of reactive oxygen species [39]. Both could diminish tissue damage caused by polymorphonuclear neutrophils (PMN) in septicaemia and/or malaria, where high serum levels of TNF and sCD14 are present.

In this respect, clinicians used pentoxifylline as a supportive agent in human malaria [14]. In an open trial in Burundian children with severe malaria, pentoxifylline exhibited a dramatic protective effect, as it significantly reduced the duration of coma, and even mortality [26]. Whether antagonism of CD14 could have an additional protective effect is not known.

In conclusion, high levels of sCD14 were found in complicated *P. falciparum* malaria, with the highest levels in patients with renal failure. The functional consequences of the elevated sCD14 concentrations could be an aggravated vascular inflammation and should be further investigated.

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