Human neutrophil lipocalin: a specific marker for neutrophil activation in severe Plasmodium falciparum malaria

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Abstract

We have earlier indicated neutrophil activation in severe malaria by measuring myeloperoxidase (MPO) and lysozyme, leukocyte granule proteins secreted by neutrophils as well as by other blood cells (monocytes/macrophages). In this study we evaluated the plasma levels of human neutrophil lipocalin (HNL), a specific neutrophil granule protein, in relation to previously reported markers MPO and lysozyme, for clinical significance in indicating severe malaria. For this purpose, plasma samples were analyzed from 65 individuals with severe malaria, mild malaria or malaria negative, all living in the Gedarif area of Sudan. The plasma levels of HNL were significantly higher in the group of patients with severe malaria as compared with the other two groups. Plasma levels of HNL correlated significantly to those of MPO and lysozyme, as well as to body temperature, degree of parasitaemia and pulse rate. These results confirm our previous findings that neutrophils are activated in-patients with severe malaria and the level of HNL is a good marker in this context.

Keywords: Severe malaria; Neutrophil; HNL; Lysozyme; MPO

1. Introduction

Plasmodium falciparum malaria remains one of the leading causes of morbidity and mortality in the tropical and subtropical regions of the world.

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The adhesion of the parasitized erythrocytes to endothelial cells, their binding to non-infected erythrocytes (rosetting) and their agglutination (auto-agglutination) are mainly mediated by *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) expressed on the surface of the infected erythrocytes (Wahlgren et al., 1999). Excessive production of tumor necrosis factor-alpha (TNF-α) by monocytes/macrophages plays a key role in the pathogenesis of both human and experimental cerebral malaria (Kwiatkowski and Perlmann, 1999). In the *P. berghei* mouse model for cerebral malaria, although neutrophils are not sequestered in the brain, depletion of neutrophils decreased the expression of TNF-α, IFN-γ and IL-12 in the brain. Therefore, it was suggested that neutrophils may play a role in the pathogenesis of experimental cerebral malaria by enhancing pro-inflammatory cytokines in the brain (Chen et al., 2000).

Involvement of neutrophils in parasite neutralizing immune responses is indicated from the observation of phagocytosis of *P. falciparum* merozoites by neutrophils in infected individuals (Kumaratilake et al., 1992). Studies have shown that the process of merozoite phagocytosis requires the presence of cytokines (e.g. TNF-α) and opsonizing antibodies (Kumaratilake et al., 1990, 1996). Neutrophils might be stimulated either directly by the parasites or by cytokines or other mediators produced during the malaria attack. Ligation of TNF-α to its receptors on neutrophils primes the cells for increased release of granule secretory products upon activation, including lysosomal enzymes/proteins (Klebanoff et al., 1986; Shalaby et al., 1987).

A role for neutrophil activation in the pathophysiology of severe malaria was indicated in our previous report by the presence of elevated levels of myeloperoxidase (MPO) and lysozyme, both acting as markers for neutrophil activation (Mohamed et al., 1996). However, both MPO and lysozyme are produced also by monocytes/macrophages. In this report we confirm our previous findings on neutrophil activation in severe malaria patients by measuring human neutrophil lipocalin (HNL) as a specific marker for neutrophil activation (Xu et al., 1994a).

2. Material and methods

2.1. Study area and subjects

The study was conducted at the Gedarif Hospital in the eastern part of Sudan, 380 km from the capital (Khartoum). Malaria in the area is markedly seasonal, and follows the annual June–September rains, with a peak of transmission during October and November. *P. falciparum* is the main parasite species, accounting for more than 96% of infections, while 3% are attributed to *P. vivax*.

An out-patient clinic was established in the hospital during the season of 1996 to serve patients suspected of having malaria infection. Full information was obtained from patients including age, sex, occupation, area of residency and patient complaints. Parasitological and detailed medical examinations (including body temperature) were carried out in all suspected malaria cases complaining of fever or giving a recent history of fever (within the past 3 days). Thick blood films were stained with Giemsa and examined for malaria parasites by counting against 200 leukocytes. Parasitaemia was calculated using an assumed white blood cell count 6000/μl. Parasitaemia was graded as low (+ = 1–999/μl), moderate (++ = 1000–9999/μl), high (+++ = 10000–99 000/μl) or very high (++++ = > 100 000/μl).

The severe malaria patients represented most of the admissions at daytimes during the study period, all of which were young adults or older children. Probably the smaller children were brought to the hospital during the night or were taken to the many health centers in the area, and were, thus, missed to be included in the study.

Three groups of patients were enrolled in the study after informed consent. The subjects of group I (*n* = 18) were those with negative blood films for *P. falciparum* and who did not show any clinical symptoms of malaria. Group II (*n* = 25) comprised patients with mild malaria and positive blood films for malaria asexual stages. Group III (*n* = 22) were those patients with positive blood films for malaria parasite together with features (hyperparasitaemia, hyperthermia, convulsions or vomiting with dehydration) of severe disease. All
malaria patients were *P. falciparum* infected and patients infected with *P. vivax* or with mixed infections were excluded. Patients and residents in the area are considered to be semi-immune. None of the patients fulfilled the World Health Organization (WHO) criteria for cerebral malaria (World Health Organization, 1990). Patients with mild, uncomplicated malaria received standard chloroquine treatment of 25 mg/kg body weight in divided doses, pyrimethamine/sulfadoxine tablets (Fansidar) or quinine tablets 10 mg/kg body weight eight hourly for 10 days. Patients with severe malaria were treated in the hospital with quinine injected intravenously 10 mg/kg body weight eight hourly changed to oral treatment as appropriate for 10 days.

Before starting treatment, 2 ml of blood were collected in EDTA tubes from all individuals involved in the study. Plasma was separated and kept frozen at −20 °C until used.

### 2.2. Measurement of plasma concentration of HNL, MPO and lysozyme

The plasma concentrations of lysozyme and HNL were measured by a double-antibody radioimmunoassay (RIA) described in detail elsewhere (Xu et al., 1994b). Briefly, 50 μl of either sample or standard was mixed with 50 μl of specific antilysozyme/anti-HNL antibodies, diluted in assay buffer, and incubated for 3 h at room temperature. Thereafter, 2 ml of decanting suspension containing Sepharose anti-rabbit IgG was added and the incubation continued for 30 min at room temperature. Lysozyme- or HNL-antibody complexes bound on Sepharose anti-rabbit IgG were separated and pelleted by means of centrifugation for 10 min at 4000 rpm. After decantation the radioactivity was measured in a gamma counter.

The plasma concentration of MPO was also assayed by means of a double-antibody RIA (Pharmacia Upjohn, Diagnostics AB, Uppsala, Sweden).

### 2.3. Statistical analysis

Parametric and non-parametric statistical methods were used for the comparisons and correlation of variables. *P* value < 0.05 was considered significant. The statistical calculations were carried out using the software Statistica (StatSoft. Inc., Tulsa, OK, USA).

### 2.4. Ethical considerations

Patients who consent to participate in the study after adequate sensitization on the project objectives and benefits were enrolled in the study. The study was monitored and supervised by qualified medical personnel whose first responsibility is the welfare of the patients enrolled in the study. The study was carried out following the WHO Guidelines for Good Clinical Practice. Ethical clearance for the study was obtained from the ethical committee at the Faculty of Medicine, University of Khartoum and from the Sudanese Federal Ministry of Health.

### 3. Results

In this study, the plasma levels of HNL, MPO and lysozyme were evaluated for their clinical significance in patients with severe malaria (group III) as compared with those with mild malaria (group II) or with negative blood film for malaria parasite (group I). The mean age of the 65 subjects enrolled in the study was 24 years (range 7–80) and there was no significant difference between the three study groups.

Table 1 shows the mean plasma concentrations of HNL, MPO and lysozyme. The severe malaria group showed significantly higher levels of all three proteins as compared with the mild malaria group or the group with no malaria (*P* < 0.001).

<table>
<thead>
<tr>
<th>Group</th>
<th>HNL (μg/l)</th>
<th>MPO (μg/l)</th>
<th>Lysozyme (μg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>73 (18.5)</td>
<td>336 (117)</td>
<td>1465 (287)</td>
</tr>
<tr>
<td>Group II</td>
<td>87 (29.3)</td>
<td>416 (138)</td>
<td>1477 (261)</td>
</tr>
<tr>
<td>Group III</td>
<td>283* (172)</td>
<td>1299* (618)</td>
<td>2275* (658)</td>
</tr>
</tbody>
</table>

Means and (S.D.) are provided. *P* < 0.001.
There were no significant differences between the mild malaria group and the group with no malaria ($P > 0.05$). There was a highly significant correlation between the plasma levels of HNL and MPO ($r = 0.82, P < 0.001$) (Fig. 1b) or lysozyme ($r = 0.75, P < 0.001$) (Fig. 1a), respectively. With regard to clinical parameters, the severe malaria group had significantly higher body temperature
(P < 0.001) and pulse rate (P < 0.001) as compared with the mild malaria group or the group with no malaria (Table 2). The plasma levels of HNL were also correlated with the degree of parasitaemia (r = 0.43, P < 0.001), body temperature (r = 0.57, P < 0.001) and pulse rate (r = 0.37, P < 0.01).

4. Discussion

The present study confirms and extends previous reports on the activation of neutrophils as a clinical parameter in severe malaria. We previously indicated neutrophil activation in severe malaria by the elevated levels of MPO and lysozyme in this patient group as compared with patients with mild malaria (Mohamed et al., 1996). However, as both MPO and lysozyme are also present in monocytes/macrophages, these cells may have contributed to the elevated levels of these proteins observed in plasma of patients presenting with severe malaria. As the lipocalin assayed in this study is specific for neutrophils (Xu et al., 1994a), our results indicate that the previously shown elevated MPO and lysozyme were also produced by activated neutrophils. We show in this study that also HNL plasma levels are highly elevated in severe malaria patients as compared with those with mild malaria or those with negative blood film for malaria parasite. The strong correlation between plasma levels of HNL, and MPO and lysozyme indicates that while monocytes/macrophages are the main producers of MPO and lysozyme, their production is correlated with the production by neutrophils in the context of malaria.

Several clinical features and laboratory parameters are currently used to define and predict falciparum malaria disease severity (Chiwakata et al., 2000; World Health Organization, 1990). Peripheral blood parasitaemia is the single best parameter of disease severity and is regarded as the reference standard, although it may not adequately reflect the total number of parasites involved in the pathophysiological process. A varying proportion of erythrocytes containing mature forms of falciparum parasites may sequester from the peripheral circulation by cytoadherence to capillary and post-capillary venular endothelium of vital organs (MacPherson et al., 1985). Activation of neutrophils directly by the malaria parasite or by cytokines (TNF-α) may result in secretion of lysosomal enzymes including human neutrophil elastase (HNE). In P. falciparum malaria, elevated plasma levels of HNE correlate with high levels of circulating thrombomodulin (Hemmer et al., 1994), which suggests neutrophil-induced vascular damage in vivo. In addition, HNE efficiently degrades coagulation factor XIII (Klingemann et al., 1982), which normally stabilizes fibrin clots by cross-linking fibrin monomers (Board et al., 1993). Both vascular damage, which may result in increased capillary permeability, and degradation of factor XIII may be induced by HNE and they may contribute to severe malaria. The low levels of factor XIII observed in malaria patients were shown to be inversely correlated not only with parasitaemia, TNF-α levels, and disease severity, but also with elevated levels of HNE which reflect neutrophil activation. In the present study, plasma levels of HNL showed correlation with the degree of parasitaemia and fever (body temperature). Other studies have shown that the pigment-containing neutrophil count is a simple marker of

<p>| Table 2 |
| Body temperature (°C), pulse and respiratory rates in group I (patients with negative blood film for P. falciparum), group II (mild malaria patients) and group III (severe disease patients) |</p>
<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Temperature</th>
<th>Pulse rate</th>
<th>Respiratory rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>30 (14–80)</td>
<td>37.3 (0.9)</td>
<td>82 (8)</td>
</tr>
<tr>
<td>Group II</td>
<td>22 (7–45)</td>
<td>37.3 (2.7)</td>
<td>96 (21)*</td>
</tr>
<tr>
<td>Group III</td>
<td>22.3 (8–50)</td>
<td>39.1 (1.3)**</td>
<td>112 (18)**</td>
</tr>
</tbody>
</table>

Mean and (S.D.) or mean and (range) are provided. *P < 0.05; **P < 0.001.
disease severity in childhood malaria in addition to the parasite count (Amodu et al., 1998).

Patients with severe malaria are vulnerable to bacterial infections and bacteraemias are an important complication of *P. falciparum* malaria in African children (Berkley et al., 1999). In this study we could not exclude concomitant bacterial infections as the cause for the elevated neutrophil markers among our patients, as concomitant infections might affect the clinical manifestation of malaria.

The activation of neutrophils would influence the clinical presentation in different ways: first directly by degranulation and producing toxic substance which will lead to tissue injury known to occur in many inflammatory diseases (Xu et al., 1994b). Second indirectly by affecting the production of TNF-α which is a known active agent in the pathogenesis of severe malaria (Schofield et al., 1993).

In conclusion, our study has clearly demonstrated that neutrophil activation occurs in severe malaria and suggested a possible role for neutrophil activation in the pathogenesis of severe malaria.

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References


