Prognostic Value of Anti-*Plasmodium falciparum*-Specific Immunoglobulin G3, Cytokines, and Their Soluble Receptors in West African Patients with Severe Malaria

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Forty-one African patients suffering from clinically defined severe malaria were studied in the intensive medical care unit of the main hospital in Dakar, Senegal, West Africa. All of these individuals lived in Greater Dakar, an area of low and seasonal *Plasmodium falciparum* endemicity. Twenty-seven patients (mean age ± 1 standard deviation, 19.2 ± 12.7 years) survived this life-threatening episode, but 14 (30.8 ± 16.2 years old) died despite initiation of adequate treatment. On the day of admission (day 0) and 3 days later, one to two blood samples (i.e., approximately 10 to 15 ml) were obtained from each subject, and different biological parameters were evaluated in the two groups. Plasma samples were tested for their content in tumor necrosis factor alpha (TNF-α), soluble receptors I and II for TNF-α (TNF-α sRI and TNF-α sRII), interleukin-6 (IL-6), IL-6 sR, IL-10, and IL-2 sR. The concentrations of all these cytokines and/or their receptors was significantly elevated in patient plasma samples on day 0, and it rapidly decreased in the group of individuals who survived. By comparison, the mean concentration of the same parameters decreased slowly in the group of patients who died (except for IL-10, which dramatically fell in all patient plasma samples soon after initiation of antimalarial treatment). The TNF-α sRI level remained significantly elevated among the patients who died, and the highest levels of soluble TNF-α sRI receptor were found among the older patients. Parasite-specific immunoglobulin M (IgM), total IgG, IgG1, IgG2, IgG3, and IgG4 were evaluated by enzyme-linked immunosorbent assay using a crude extract of a local *P. falciparum* isolate as antigen and human class- and subclass-specific monoclonal antibodies. Parasite-specific IgM, total IgG, and IgG1 were detectable in the plasma samples of most of these African patients, whereas IgG2 and IgG4 mean values were low. The mean level of parasite-specific IgG3 was different (P = 0.024) at day 0, i.e., before initiation of intensive medical care, between the group of the 27 surviving subjects and the group of 14 patients dying of severe malaria. As a consequence, most of the African patients who died had only trace amounts or almost no detectable level of parasite-specific IgG3 at the time of admission. In contrast, the presence of even limited IgG3 activity at day 0 was found to be associated with a significantly increased probability of recovering from severe malaria. Therefore, in our study, both an elevated level of TNF-α sRI and absence of IgG3 activity were of bleak prognostic significance, whereas a favorable outcome was usually observed when parasite-specific IgG3 activity was detectable. This finding was strongly suggestive of a prime role for these parasite-specific immunoglobulins in the capacity to help recovery from severe malaria.

The most dramatic evolution of *Plasmodium falciparum* infection is the development of severe malaria. The precise mechanisms leading to this often fatal outcome are far from being clearly understood, and a number of different pathological signs have been found associated with this form of the disease (35, 36). It has been suggested that cytoadherence in cerebral capillaries was involved, and severe malaria was confirmed postmortem in apparently aparasitemic children (34). In addition, erythrocyte rosetting was also suspected of playing a critical role in human severe malaria (5). Nevertheless, both cytoadherence and rosetting could be only part of the process, as, for example, T-cell responsiveness was found to be altered in severe malaria (3, 12). Moreover, the level of TNF-α was found to be associated with the severity of *P. falciparum* attacks (16) and also with the plasma interleukin-6 (IL-6) and gamma interferon concentrations (23). A quite common belief is that severe malaria affects more often young individuals (i.e., relatively naive patients), as in areas of endemicity, most of the fatal cases are found among children. This is only partially true, and we have recently emphasized that African adults can also suffer from severe malaria (30). In addition, it was recently demonstrated that severe malaria in Gambian children was not due to a lack of previous exposure to malaria (13). Hence, exposure alone is apparently not sufficient to avoid severe malaria attacks, and both African children and adults living in areas of endemicity, i.e., individuals previously exposed to *P. falciparum* infection, do occasionally develop severe malaria. For this reason, and in addition to the measure of some prognostic cytokines, we have also evaluated, in the same patients, to what extent the initial exposure to *P. falciparum*, namely, the parasite-specific antibody subclass-associated responses, could interfere with the evolution of clinically defined severe malaria episodes.

In this work, we show that severe malaria can be found in both young and adult African patients. Most of these individ-
TABLE 1. Frequencies of some clinical and biological criteria associated with cerebral malaria among studied patients

<table>
<thead>
<tr>
<th>Criterion</th>
<th>No. tested</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCS &lt;7</td>
<td>8/41</td>
<td>19.50</td>
</tr>
<tr>
<td>Parasitemia &gt;5%</td>
<td>7/41</td>
<td>17.10</td>
</tr>
<tr>
<td>Convulsions</td>
<td>9/41</td>
<td>21.95</td>
</tr>
<tr>
<td>Severe anemia</td>
<td>1/40</td>
<td>2.50</td>
</tr>
<tr>
<td>pH &lt; 7.25</td>
<td>5/41</td>
<td>12.19</td>
</tr>
<tr>
<td>HCO₃ &lt; 15 mmol/liter</td>
<td>6/41</td>
<td>14.63</td>
</tr>
<tr>
<td>Glucose &lt; 2.2 mmol/liter</td>
<td>3/41</td>
<td>7.32</td>
</tr>
<tr>
<td>Bilirubin &gt; 59 μmol/liter</td>
<td>15/40</td>
<td>37.50</td>
</tr>
<tr>
<td>Arterial blood pressure &lt; 50 mm Hg</td>
<td>3/41</td>
<td>7.32</td>
</tr>
<tr>
<td>Creatinine &gt; 0.265 μmol/liter</td>
<td>15/41</td>
<td>36.60</td>
</tr>
</tbody>
</table>

* Numbers and percentages of cerebral malaria patients with unarousable coma, hyperparasitemia, convulsion, acidosis, hypoglycemia, jaundice, collapse, and renal failure.

Mathematical and materials

The 41 patients were all individuals of African origin, living in Greater Dakar, and of severe cerebral malaria transmission (26). They were enrolled in the study immediately on arrival at the emergency unit of the main hospital in Dakar (Hôpital Principal). All patients enrolled in the study displayed the same pattern of infection as already described for the Hôpital Principal by the same staff of medical doctors. In particular, all criteria for inclusion of the patients as having severe malaria were in agreement with published observations of the pathophysiology of severe falciparum malaria (9, 26).

The diagnosis of severe malaria was established by World Health Organization criteria (31) and was assessed by trained staff of the hospital's emergency unit. Severe malaria patients were defined on the basis of the confirmed presence of a positive blood smear or quantitative buffy coat visualization of acidine orange-stained parasites, anemia, neurological signs, and renal or hepatic failure (Table 1). The degree of consciousness was assessed by the Glasgow coma scale (GCS), which varied between 3 and 15, an unrousable coma and for certain patients 12 days later for plasma content analysis in the Institut Pasteur facilities in Dakar. Each of the 41 patients included in the study was thus sampled at least once, on the day of admission (day 0), i.e., before initiation of intensive medical care; 32 patients were blood sampled a second time between 2 and 4 days after admission (mean = 3.2 ± 0.9 days, referred to hereafter as day 3). This protocol had received the agreement of the hospital's ethical committee before the initiation of the study.

A total of 74 different samples were obtained. As soon as the blood was drawn, the Vacutainer tube was centrifuged; the plasma was aliquoted in 200-μl endo-

Cytokine level evaluation. Serum levels of TNF-α, TNF-α sRI, TNF-α sRII, IL-2 sRI, IL-6 sRI, and IL-10 were evaluated by ELISA, using commercially available Medgenix EASIA kits from Medgenix Diagnostics S.A. (Fleurus, Belgium) as instructed by the manufacturer. In brief, samples or standards containing cytokines react with capture monoclonal antibodies (MAbs 1) coated on the microtiter well and with a MAb (MAb 2) labeled with horseradish peroxidase.

Antigen preparation. A local Senegalese isolate recently obtained in the vil-

Bacteriological parameters. Daily parasitemia was determined by counting the number of axenial Plasmodium forms per 1,000 erythrocytes. The actual level of parasitemia was estimated by using the total erythrocyte count, and all necessary biological determinations for intensive care follow-up were performed in the hospital facilities.

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correspond to the difference observed between the OD levels actually measured and the background OD values.

**RESULTS**

**Cytokines and their receptors found at the time of admission.** Of the 41 patients (mean age ± 1 standard deviation [SD], 22 ± 14 years; range, 1 to 58 years), 27 had a satisfactory evolution whereas 14 died of severe malaria. The age factor appeared to be potentially critical in our study group, as the mean age of the surviving patients (19 ± 12 years) was lower (P = 0.041) than that of the patients who died (30 ± 17 years). The patients enrolled in this study included 12 children under 15 years of age (2 of them died) and 29 individuals over 15 years of age (12 of them died).

The level of parasitemia evaluated on the day of admission (day 0) was extremely variable but remained comparable between the two groups (65,641 ± 92,107 [mean ± 1 SD] and 75,168 ± 93,131 [mean ± 1 SD] parasites/μl in the groups of patients who survived and those who did not, respectively). No relationship was found between parasitemia and either TNF-α or IL-6, whereas a strong correlation was observed with IL-2 sR (r = 0.328, P = 0.004), TNF-α sRI (r = 0.363, P = 0.02), TNF-α sRII (r = 0.421, P < 0.01), and IL-10 (r = 0.567, P < 0.001).

At day 0, the mean levels of TNF-α were different in the two groups (P < 0.05), and the mean levels of soluble receptors for this cytokine were also markedly different (P < 0.001 and P < 0.01 for TNF-α sRI and TNF-α sRII, respectively), as illustrated in Table 2. The mean levels of TNF-α, TNF-α sRI, and TNF-α sRII were higher (1.55-, 2.60-, and 1.61-fold, respectively) in the group of patients who died than in the group who survived. In addition, the level of TNF-α sRII was correlated with age at day 0 (r = 0.42, P = 0.008). In contrast, IL-6 (+122%), IL-6 sR (+114%), IL-10 (+189%), and IL-2 sR (+125%) levels were elevated but not significantly different between these two groups at the time of enrollment, i.e., before initiation of intensive treatment (Table 2).

**Cytokines and their receptors found after initiation of treatment.** Three days after admission and initiation of treatment, the level of peripheral parasite density had dramatically decreased in all patients, indicating that the antimalarial treatment by parenteral injection of quinine was efficient.

Most mean levels of plasmatic cytokines and their soluble receptors decreased in the successfully treated patients. Levels of TNF-α, TNF-α sRI, TNF-α sRII, and IL-2 sR (all with P < 0.03) and IL-10 (P < 0.001) were markedly lower at day 3 than at day 0 among the survivors (Table 2).

The level of TNF-α and that of its soluble receptor remained significantly higher among the patients who died than among the survivors (i.e., +322%, +383%, and +259% for TNF-α, TNF-α sRI, and TNF-α sRII, respectively). After 3 days of treatment, the level of TNF-α sRII still remained highly correlated with age (r = 0.615, P < 0.001).

The mean level of IL-2 sR was 214% higher in the group of patients who died than in the group of patients who recovered. As illustrated in Table 2, the difference was mainly due to the rapid and striking decrease in the mean IL-2 sR level found among the surviving patients (1.65-fold decrease) compared to the persistently sustained mean level of IL-2 sR found in the group of patients who died. Whereas this was not the case on the day of admission, the IL-2 sR levels were correlated with age at day 3 (r = 0.476, P < 0.01).

IL-6 was extremely high after 3 days of treatment, with a mean value of +218% in the group of patients who died compared to that in the group of survivors. No significant difference could be detected for IL-10 between the two groups of patients (Table 2). Among the patients tested up to day 7, the level of cytokines showed the same trend toward reduced values (data not shown).

**Parasite-specific immunoglobulin responses found in the different groups of patients.** At the time of admission, the parasite-specific immunoglobulin OD values were compared between the two groups of patients with severe malaria. As shown in Fig. 1, no difference could be detected between the groups of survivors and those who died for the mean parasite-specific antibody classes (IgM and total IgG). No difference in the parasite-specific subclass values was found for IgG1, IgG2, and IgG4. In contrast, on the day of admission, i.e., before treatment, the level of parasite-specific IgG3 subclass activity differed between these two groups (P = 0.024). The mean parasite-specific IgG3 OD values were ninefold higher among the surviving patients than in the group who died, who had almost no detectable trace of IgG3 on the day of admission. Limited but detectable IgG3 OD values were exclusively found in the group of surviving patients (Table 3).

**DISCUSSION**

In this study, we investigated not only the plasma levels of several different immunoregulatory molecules but also the parasite-specific antibody activity found on admission. The group of 41 patients suffering from severe malaria was composed of African inhabitants of Greater Dakar, an area of low and seasonal malaria transmission. Severe malaria attacks in Sene-
who died. Specific IgG3 activity between the group of survivors and the group of patients plasma at the time of admission. A significant difference was detectable for mean standard error of the mean) distribution in severe malaria patient plasma at the time of admission. A significant difference was detectable for specific IgG3 activity between the group of survivors and the group of patients who died.

gal were previously found both in nonimmune Caucasian adult patients with no trace of parasite-specific antibodies and in African adults (33).

The patient plasma contents were tested for a number of potential biological markers of complicated P. falciparum malaria, particularly TNF-α and its receptors (7, 8, 19, 21, 22). The levels of the two receptors for TNF-α were extremely high and at day 0 and remained so later on, particularly those of type I, among the patients who died. A relationship between TNF-α receptor levels and severity of the disease was therefore clearly apparent in our work as previously found in previous studies on malaria (10, 18, 20).

The difference in cytokine levels between the two groups of patients was mainly due to a general tendency for a marked decrease in the mean values found among those who recover. In contrast, a sustained level of the same parameters was observed among the patients who died, as previously described (6, 14, 32). As a result, the mean values observed at day 3 for TNF-α, the two receptors of TNF-α, IL-10, IL-6, and IL-2 sR were not significantly different from those found at day 0 in the group of patients who died despite intensive care.

The 41 patients were tested by ELISA for P. falciparum-specific antibody activity. This parasite-specific antibody activity was previously tested in different malaria-endemic areas of Senegal with the same isolate, and for a given individual, it was found to be relatively stable even at an interval of a few months (1). This was again the case here, as no major change in the total antibody content was detectable during this short period of follow-up. In the present study, age and/or parasitemia were not found to be directly associated with a given pattern of isotypic distribution. Therefore, the pattern of parasite-specific antibody response was probably much more indicative of previous exposure to parasite transmission than of the attack necessitating treatment.

The parasite-specific IgM levels were slightly, but not systematically, increased after admission, and they remained elevated for up to day 7 (data not shown). Such changes probably reflected a boosting of the host immune response in the presence of a new strain responsible for, or a new set of antigenic determinants associated with, the clinical episode.

It is noteworthy that the total IgG content was not predictive of the future evolution of the patients with severe malaria at either the group or the individual level. It was only a rough indication of previous exposure to the parasite in Greater Dakar. IgG1, IgG2, and IgG4 activities (only trace amounts of the latter were detected among the patients) were comparable in the two groups of patients with severe malaria. In contrast, whereas almost no trace of parasite-specific IgG3 activity was detectable among the patients with fatal evolution, low but consistent activity of this same isotype was found in the group of patients with favorable evolution. Therefore, the parasite-specific IgG3 activity which was already found to be associated with a reduced susceptibility to clinical malaria attack in a malaria-holoendemic area of Senegal (1) was also found to be associated with a lowered risk of death following severe malaria attack in Senegalese patients.

That severe malaria affects people previously exposed to P. falciparum was previously demonstrated among Gambian children (13), but in a study conducted in Senegal (28), no clear-cut relationship between antibody level and the degree of severity of the disease was observed. Nevertheless, the final issue of a clinical episode was also reportedly dependent on the level of fluorescent antibodies at the time of admission. In contrast, Oudart et al., working in the same area, did not find a correlation between the severity of malaria and the level of blood antibodies (25). Therefore, past history of exposure to the parasite (hence antiparasite total IgG antibody per se) clearly does not systematically protect a given individual. In Thailand, Brasseur et al. found that immunofluorescent antibody titers significantly differed between patients who died of severe malaria despite adequate therapy and those who recovered (4).

These authors suggested that antibody production may be required to achieve recovery with drug treatment in severe malaria, and this could be the case in our study also.

In the present study, the parasite-specific IgG3 subclass could have played a critical role in helping achieve recovery.
from severe malaria in conjunction with antimalarial therapy. This is indeed reminiscent of several passive transfers in human patients and associated studies illustrating a crucial role for cytotoxic antibodies. Since the total parasite-specific IgG levels were comparable for all patients, the need for qualitative and not quantitative parasite-specific humoral response is clearly emphasized here and tends to confirm a number of previous observations (1, 2, 11, 17, 24). This is indeed reminiscent of experiments pointing out a particular role for the cytotoxic antibody subclasses in the mechanisms of defense against Plasmodium falciparum attacks. In addition to this protective role, other possibilities include a potential interaction with cytokines and/or their receptors remained markedly elevated the dramatic parasite burden.

The capacity to recover from severe malaria was obviously not associated with a better initial control of the circulating parasitemia, as this parameter was largely comparable, on the day of admission, between the two groups of patients. It must be stressed that to some extent, the peripheral parasitemia was apparently controlled before day 3 among a number of individuals who ultimately died with no detectable parasitemia (data not shown). This could have reflected a pronounced tendency for deep sequestration in these latter individuals. As the parasite load was extremely elevated, secondary effects of the parasite-specific antibodies: the IgG3 isotype could have efficiently controlled in the presence of a minimal activity of such heavy parasitemia, as this parameter was largely comparable, on the other side, to rapidly regulate the level of different plasma cytokines.

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