Mannose-Binding Lectin Plasma Levels and Gene Polymorphisms in Plasmodium falciparum Malaria

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The contribution of mannose-binding lectin (MBL) to protection from malaria was assessed by comparing plasma concentrations of MBL and the frequency of MBL gene polymorphisms in groups of Gabonese children participating in a prospective study of severe and mild malaria due to infection with Plasmodium falciparum. At admission, a higher proportion of patients with severe malaria had a low level of MBL compared with subjects with mild malaria (0.35 vs. 0.19, P = .02). Two mutations in codons 54 and 57 of the MBL gene were detected. They were present at higher frequency in those with severe malaria (0.45 vs. 0.31, P = .04). These results suggest that deficient innate immune responses, in the form of low MBL levels, may be a risk factor for severe malaria in some young children who lack well-developed, clinically protective acquired immune responses.

More than 1 million children die every year from severe malaria in areas of sub-Saharan Africa where Plasmodium falciparum is endemic [1]. In such areas, effective clinical immunity develops only slowly after birth, and the burden of risk of developing severe disease is thus carried by young children [2]. These observations have prompted the search for mechanisms of protection, and innate resistance or susceptibility to severe malaria has thus recently been linked to genetic factors influencing immune responsiveness [3, 4].

Mannose-binding lectin (MBL) is a member of the collectin family of proteins, which are constituents of the innate immune system [5]. MBL mediates its effect through carbohydrate recognition domains with multiple specificities for sugar residues on microbial surfaces, resulting in opsonization via collectin receptors and/or complement activation [6]. MBL is thought to be most effective at an early age, before effective acquired immune responses have developed, acting as an ante-antibody, and MBL deficiency has thus been linked to chronic susceptibility to infection in children [7]. Low plasma concentrations of MBL result primarily from mutations in the coding region of the MBL gene, causing structural alterations in the collagenous region of the molecule, rendering the protein nonfunctional due to its inability to form stable oligomers [6].

We determined, by comparing the plasma levels of this protein and the MBL gene polymorphisms in individuals with either severe or mild malaria, whether MBL may influence the response to infection with P. falciparum in young African children. In the study area, the majority of malaria cases requiring hospital care present with severe anemia, hyperparasitemia, or both. Both of these parameters can be considered to be markers of uninhibited parasite multiplication, which seems to be a prerequisite for a fatal outcome of malaria in this setting.

Methods

Study subjects. The patients were from a matched-pair case-control study of severe malaria undertaken in Albert Schweitzer Hospital, Lambarene, Gabon. The study was cleared by the Ethics Committee of the International Albert Schweitzer Foundation. Informed consent was obtained from the parent or guardian of each child. The study was supported by the Albert Schweitzer Foundation and grant No. 19990089 from the German Research Foundation (DFG).

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Hospital in Lambaréné, Gabon, which has been described in detail elsewhere [8]. Children (n = 100) with severe malaria fulfilled the following inclusion criteria: P. falciparum malaria with >1000 parasites/lL and older than 6 months, with severe malaria defined as severe anemia (hemoglobin <50 g/L) and/or hyperparasitemia (>250,000 parasites/lL corresponding to >10% infected erythrocytes) and facultatively other signs of severe malaria [9]. As controls, sex-, age-, and provenance-matched children with mild malaria were admitted as soon as possible after each severe malaria patient. The following criteria for the mild malaria controls had to be fulfilled: P. falciparum malaria with a parasitemia of 1000–50,000/lL on admission, no schizontemia, malarial pigment-containing circulating leukocytes <50/lL, hemoglobin >80 g/L, platelets >50/nL, leukocytes <12/nL, lactate <3 mmol/L, blood glucose >50 mg/dL, no signs of severe malaria or other acute infections, no intake of antimalarial drugs within the preceding week, and no history of hospitalization (to exclude prior severe malarial attacks).

All 200 children were monitored in the acute phase of the disease, and after treatment they became subject to an ongoing, longitudinal, prospective study to compare reinfection rates with immune responses and other factors. Routine pediatric micromethods were used to measure hematologic and biochemical parameters and parasitemia [8].

**MBL measurements.** Venous blood from study participants was collected into heparinized tubes at admission, after 1 month, during convalescence, and again after at least 6 months, when the children were healthy. Plasma was collected after centrifugation of blood and stored at −80°C. MBL was quantified in plasma samples by EIA according to a published method [10], using a monoclonal anti-MBL antibody for capture and a biotinylated anti-MBL monoclonal antibody for detection (both from Statens Serum Institut, Copenhagen), followed by extravidin-peroxidase (Sigma, Deisenhofen, Germany), and tetramethylbenzidine substrate (Dunn Laborteknik, Aschab, Germany). The cutoff for this assay was 20 µg/L.

**MBL gene polymorphisms.** Polymorphisms in the MBL gene were detected by polymerase chain reaction (PCR) and DNA sequencing. PCR was performed on a RapidCycler (Idaho Technologies, Idaho Falls, ID). The reaction conditions were 92°C for 5 s, 50°C for 5 s, and 72°C for 15 s. The PCR-generated DNA fragments were separated and analyzed using 1.2% agarose gel electrophoresis according to standard procedures. The sequences of the primers used were MBL-1: 5'-GTAGGACAGGGCATGCTC-3' and MBL-2: 5'-CAGGCAGTTTCTCTGGAAGG-3'. Primers were purchased from Interactiva (Ulm, Germany). The genotype was initially determined by DNA sequence analysis on a sequencer (ABI; Applied Biosystems, Perkin-Elmer, Foster City, CA). After 120 samples were sequenced and only the known mutations in codons 54 and 57 were found, we determined the remaining samples by restriction analysis as previously described [11].

**Statistics.** Contingency tables, using the McNemar test, were used to compare proportions between the groups. Nonparametric statistical methods were used to detect differences between continuous variables. The Wilcoxon signed rank test was used for pairwise comparisons. Significance of differences was set at a two-tailed P < .05. The 25th percentile level (200 µg/L) of MBL concentration in the 6-month samples, taken when the children were healthy, was used as a cutoff to define a low plasma concentration of MBL. MBL concentrations are expressed as median ± median absolute deviation in micrograms per liter of plasma.

**Results**

**Clinical investigation.** The matched patient groups comprised 61 female and 39 male subjects, with a mean age of 44 ± 2 months. Detailed clinical and parasitologic data have been presented elsewhere [8]. Three individuals with severe complications died within 2 days of admission. Treatment resulted in parasitologic and clinical cure in all other patients. Parasitemia (307,000 ± 11,500/µL vs. 10,650 ± 8500/µL in severe vs. mild, respectively) and hematocrit (21.6 ± 0.6 vs. 32.9 ± 0.4, respectively) differed significantly (P < .001) between the groups. As expected, there was a significantly higher proportion of those with mild malaria who had the sickle-cell trait (0.21 vs. 0.10, P < .05).

**Plasma MBL concentrations and MBL gene mutations.** Plasma concentrations of MBL are shown in figure 1. In the acute phase of the infection, a significantly higher proportion of the severe malaria patients (0.35 severe vs. 0.19 mild, P = .02) had a low (<200 µg/L) MBL concentration. In the samples taken during convalescence (1 month) and at 6 months, the...
range of concentrations of MBL was narrower than in the acute phase, and the median values in the 2 groups were similar.

We detected two previously described mutations in exon 1 of the MBL gene, at codons 54 and 57. At least one mutation was present in 38% of the children, with an overall gene frequency of 0.02 and 0.18 for codon 54 and 57 mutations, respectively. Mutations, in either the homozygous or heterozygous state, were present at higher frequency in severe malaria patients than mild malaria controls (0.45 vs. 0.31, \( P = .04 \)). At the individual mutation level, a significantly higher proportion of severe malaria patients than mild malaria controls carried the mutation at codon 54 (0.08 vs. 0.01, \( P = .04 \)). Three individuals were homozygous for the codon 57 mutation.

Plasma levels of MBL in relation to the mutations are shown in table 1. Comparison of individuals with the wild-type MBL gene showed that severe malaria patients had significantly higher (\( P < .05 \)) MBL levels in the acute phase of the infection. In the convalescent phase, the median MBL level was still significantly higher (\( P < .05 \)) in severe than in mild malaria, but after 6 months, there was no longer any difference between the groups. The presence of a mutation in the MBL gene resulted in uniformly low MBL levels regardless of an individual’s clinical presentation, so that, at any time point, a significantly higher proportion (>85%) of those with an MBL concentration <200 \( \mu \)g/L had an MBL gene mutation (\( P < .001 \)). There was no significant difference between the groups in the levels of MBL present at the different time points in those with MBL gene mutations.

### Discussion

The principal finding of this matched-pair case-control study is that structural polymorphisms of the MBL gene, which result in low plasma concentrations of nonfunctional protein, are more likely to be found in children with severe than mild malaria. One mutation, at codon 54, was significantly more frequent in those with severe malaria.

As a component of the innate immune system, MBL has an important role to play as a first line of defense against multiple pathogenic organisms. The diversity of its potential targets arises from the variety of specificities of its carbohydrate recognition domains, through which it binds to microbial surfaces, opsonizing and/or activating complement via the lectin pathway [6]. The gene frequencies for the two mutations we found in the Gabonese population studied here are in accordance with those reported for other African populations, as is the strong association we found between low MBL levels and presence of either mutation [11].

The fact that we found more MBL gene mutations in severe malaria patients, associated with very low plasma MBL levels, raises the possibility that MBL has some role to play in protection against malaria. Since MBL binds to surface glycoproteins on protozoal parasites [5], a role could be envisaged for this protein in inhibition of \( P. falciparum \) blood-stage multiplication through, for example, binding to carbohydrate residues on the merozoite surface, thereby preventing invasion of erythrocytes. In this context, the glycosylphosphatidylinositol membrane anchor, which is a constituent of the \( P. falciparum \) merozoite surface antigen-1, for example, represents a compelling potential target for MBL binding through its multiple mannose residues [12]. There is as yet no definitive proof that MBL binds to such residues on merozoites.

Clearly, however, in our study a high plasma level of MBL was not on its own predictive of protection from severe malaria. This much is evident from the comparison of individuals with the normal wild-type MBL gene, which showed that acute-phase MBL levels were significantly higher in severe malaria patients than in mild malaria controls. We would interpret these higher levels of MBL as reflecting a more intense acute-phase response induced in part, and indirectly, by the higher parasitemias in severe malaria patients compared with mild malaria controls. In general, severe malaria is a systemic life-threatening event during which the expression of all mediators of the innate immune response is highly up-regulated. Thus, if there is no impairment of the expression of a particular molecule, the production of acute-phase proteins, inflammatory cytokines, oxygen radicals, and nitric oxide is acutely enhanced and higher plasma levels are consequently found in severe compared with

### Table 1. Frequencies of MBL gene mutations and MBL plasma concentrations according to clinical presentation.

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>No. of cases</th>
<th>Severe malaria</th>
<th>Mild malaria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time 0</td>
<td>1 month‘</td>
<td>6 months‘</td>
</tr>
<tr>
<td>A/A</td>
<td>55</td>
<td>5150 ± 3320</td>
<td>4870 ± 3090</td>
</tr>
<tr>
<td>A/B</td>
<td>6</td>
<td>165 ± 165</td>
<td>2150 ± 2150</td>
</tr>
<tr>
<td>A/C</td>
<td>35</td>
<td>0 ± 0</td>
<td>605 ± 600</td>
</tr>
<tr>
<td>B/C</td>
<td>2</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>C/C</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Any mutation</td>
<td>45</td>
<td>0 ± 0</td>
<td>570 ± 570</td>
</tr>
</tbody>
</table>

NOTE. MBL plasma values are median ± median absolute deviation in µg/L; ND, not done (samples not available).

* Genotype codes: A, wild-type; B, codon 54 mutation; C, codon 57 mutation.

‘ Sample sizes = 156 for 1 month, 105 for 6 months.
mild malaria [13]. In this context, we speculate that individuals who developed severe malaria, despite having markedly elevated plasma MBL concentrations, did so because of an impairment in other genetically or immunologically related mechanisms that mediate protection. The similarity of ethnicity profiles in the 2 groups (data not shown) excluded ethnic origin as a potential confounder in this study.

In children with the wild-type MBL gene, plasma MBL levels were maintained at concentrations above baseline (healthy) levels for at least 1 month after admission. Other studies have reported similarly prolonged elevation of MBL levels in malaria patients [14]. These findings probably reflect a persistent systemic inflammatory response associated with clearance of parasites and removal of parasite-derived metabolites, such as hemoglobin. In those with MBL gene mutations in the heterozygous state, plasma MBL levels tended to be lower at admission, in particular in patients with severe malaria. Although nonfunctional, the MBL produced in these individuals may retain carbohydrate residue-binding activity. Thus, in those with very high parasitemias, the low amounts of MBL normally present may be bound to parasite metabolites and therefore remain at low and undetectable levels.

The fact that polymorphisms in the MBL gene are maintained at high frequencies in African as well as in non-African populations suggests that low MBL levels must have some biologic advantage in certain circumstances. Less immunopathology due to reduced complement activation and protection from infection with intracellular parasites, which use complement binding and receptor uptake to infect host cells, have both been proposed as possible explanations [6]. Mycobacteria, including Mycobacterium tuberculosis and Mycobacterium leprae, bind MBL and require complement components for successful host-cell invasion [15, 16]. It could thus be argued that the high prevalence and broad global distribution of tuberculosis, and possibly leprosy, have provided the selection pressure required to ensure that the polymorphisms in the MBL gene are maintained, despite the potentially harmful consequences of low MBL levels during infection with other widespread disease agents such as Plasmodium falciparum. A similar argument has been used as a possible explanation for the maintenance at high frequency of a tumor necrosis factor-α gene promoter variant associated with susceptibility to cerebral malaria [4].

In summary, we report here an association between a common deficiency of the human innate immune system and susceptibility of some children to severe malaria. Our findings suggest that MBL might prove to be a protein to be added to the list of genetically related human factors that can influence the course of disease following infection with Plasmodium falciparum.

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10. Garred P, Madsen HO, Kurtzhals JAL, et al. Diallelic polymorphism may both nonfunctional, the MBL produced in these individuals may retain carbohydrate residue-binding activity. Thus, in those with very high parasitemias, the low amounts of MBL normally present may be bound to parasite metabolites and therefore remain at low and undetectable levels.

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