

## Mannose-Binding Lectin Is a Disease Modifier in Clinical Malaria and May Function as Opsonin for *Plasmodium falciparum*-Infected Erythrocytes

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**Variants alleles in the mannose-binding lectin (MBL) gene (*mbi2*) causing low levels of functional MBL are associated with susceptibility to different infections and are common in areas where malaria is endemic. Therefore, we investigated whether MBL variant alleles in 551 children from Ghana were associated with the occurrence and outcome parameters of *Plasmodium falciparum* malaria and asked whether MBL may function as an opsonin for *P. falciparum*. No difference in MBL genotype frequency was observed between infected and noninfected children or between children with cerebral malaria and/or severe malarial anemia and children with uncomplicated malaria. However, patients with complicated malaria who were homozygous for MBL variant alleles had significantly higher parasite counts and lower blood glucose levels than their MBL-competent counterparts. Distinct calcium-dependent binding of MBL to the membrane of *P. falciparum*-infected erythrocytes, which could be inhibited by mannose, was observed. Further characterization revealed that MBL reacted with a *P. falciparum* glycoprotein identical to the 78-kDa glucose-regulated stress protein of *P. falciparum*. MBL seems to be a disease modifier in clinical malaria and to function as an opsonin for erythrocytes invaded by *P. falciparum* and may thus be involved in sequestration of the parasite, which in turn may explain the association between homozygosity for MBL variant alleles and high parasite counts.**

Strong evidence indicates that the outcome of severe malaria may be linked to the genetic constitution of the host. Genetic variations involving red cells, for example, in the hemoglobin structure (HbS, HbC, and HbE alleles) and in the rate of synthesis of hemoglobin chains (thalassemia), are frequent in areas where malaria is highly endemic. These are classical examples of genetic variations in which homozygosity is unfavorable for the host, while heterozygosity may confer a selective advantage (heterosis) (1). Certain HLA antigens that are common in West Africans have been shown to be associated with reduced susceptibility to severe disease (22), while variations in genes causing inflammation, e.g., the gene that encodes tumor necrosis factor alpha, have been associated with a poor malaria outcome (38). In addition, genetic variations in several other candidate genes have been suggested, including the *mbi2* gene (2, 33).

Mannose-binding lectin (MBL) is a serum protein primarily synthesized by the liver and subsequently released into the bloodstream. MBL is thought to play an important role in the innate immune defense and has functional and structural similarities to lung surfactant proteins A and D, the ficolins, and C1q (5, 54). Human MBL is encoded by a single gene (*mbi2*)

on chromosome 10 (44, 51). The ligands for MBL are high-mannose and *N*-acetylglucosamine oligosaccharides present on a variety of microorganisms. MBL may activate the complement system via MBL-associated serine proteases and interact with receptors on phagocytes (37, 40, 52). In the general population, the protein has been shown to be of particular importance in protection against bacterial and viral infections during the vulnerable period of infancy between 6 and 18 months of age prior to the establishment of specific immune protection provided by the adaptive immune system (26). In addition, MBL variant alleles causing low MBL levels in serum are associated with an increased risk of different types of infections primarily in children (8, 47, 48) but also in adults with an accompanying disease or immunodeficiency (9, 49). Moreover, a significant implication of MBL deficiency in autoimmune diseases has been documented (4, 7, 10, 11, 21, 46).

Three single base substitutions in exon 1 of the *mbi2* gene independently cause low levels of MBL in serum: at codon 54 (glycine to aspartic acid, allele *B*), at codon 57 (glycine to glutamic acid, allele *C*), and finally at codon 52 (arginine to cysteine, allele *D*) (32, 34, 47). The common designation for these variant alleles is *O*, while the normal allele has been named *A*. Each of the three variants reduces the amount of functional MBL subunits in heterozygous individuals 5- to 10-fold (12). Moreover, several nucleotide substitutions in the promoter region of the *mbi2* gene affect the MBL level in serum (35, 36). In particular, a polymorphism in codon -221

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(*X/Y* type), which is exclusively situated on chromosomes not carrying the variant structural alleles, has a marked down-regulating effect on the MBL concentration in serum (12, 35, 36).

The MBL *C* allele is extremely common in sub-Saharan Africa, reaching allele frequencies of 0.30, while the *B* allele can be found in Europe (0.13), in Asia (0.20), and in indigenous people of South America, where it can reach allele frequencies of up to 0.50 (32, 34, 36). The *D* allele can be found in northeastern Africa and in Europe with allele frequencies of around 0.05 (34). Thus, it has been hypothesized that these alleles are positively selected in response to reduced susceptibility to or mortality due to certain infectious diseases (13, 14, 32).

Because these alleles are so frequent in areas where malaria is endemic and may affect the outcome of different infectious diseases, we investigated whether MBL has a role in malaria by (i) determining MBL alleles in children with and without clinical malaria and (ii) testing the notion that MBL may be an opsonin for *Plasmodium falciparum*-infected erythrocytes (RBC).

#### MATERIALS AND METHODS

**Epidemiological study.** The study population was children between 0 and 13 years old consecutively admitted with *P. falciparum* malaria to the emergency room at the Department of Child Health, Korle-Bu Teaching Hospital, Accra, Ghana, during the malaria season (June to August) in 1995, 1996, and 1997. The patient groups have been described in detail elsewhere (30). Briefly, we included patients who fulfilled strict criteria for cerebral malaria ( $n = 141$ ), severe malarial anemia ( $n = 73$ ), and uncomplicated malaria ( $n = 109$ ). All patients were febrile, with an axillary temperature of  $>37.5^{\circ}\text{C}$ . In addition, patients with severe anemia had a hemoglobin concentration of  $<50$  g/liter, no other cause of anemia, and full consciousness (score of 5 on the Blantyre coma scale) (39). Uncomplicated patients met the same criteria as those with malarial anemia but had hemoglobin levels of  $>80$  g/liter and no other complications of malaria (e.g., respiratory distress or convulsions). Patients with cerebral malaria had unrousable coma and a coma score of  $\leq 3$  for more than 60 min and no sign of meningitis or encephalitis on examination of cerebrospinal fluid. We excluded malaria patients who did not fall into the three categories, patients with any disease other than malaria, and patients with a positive test for the sickle cell gene (metabisulfite method).

Additionally, healthy, sickle cell-negative children between 5 and 15 years of age with or without detectable *P. falciparum* in thick blood films ( $n = 147$  and 81, respectively) from a nearby community, Dodowa, were included.

Both patients and controls from Dodowa were included after signed consent was given by parents or guardians who received simple standardized information in the local language. The ethics and protocol review committee at the University of Ghana Medical School and Ministry of Health, Ghana, approved the study.

Venous blood samples were drawn at admission into test tubes containing EDTA. Plasma was separated within 2 h after collection, and pellets were frozen at  $-20^{\circ}\text{C}$  until purification of DNA. We measured hemoglobin and counted white blood cells by an automated hematology analyzer. From each individual sample, thick and thin blood films were made and stained with Giemsa for microscopic detection and identification of *P. falciparum*. The number of parasites per 300 white blood cells was determined in the thick blood films, and the number of parasites per microliter with the measured white blood cell count was calculated. When parasite counts were high, at least 1,000 parasites and no more than 50 white blood cells were counted.

Genomic DNA was isolated from EDTA-treated blood cells and stored at  $-20^{\circ}\text{C}$ . DNA was amplified by general and site-directed mutagenesis PCRs, and the MBL alleles were detected as previously described (12, 35). All three structural variant alleles (*B*, *C*, and *D*) have a considerable effect on MBL concentrations, and to avoid small groups, the three alleles were grouped into one category (called allele *O*). The normal allele was designated *A*. The following six MBL genotypes were defined: the *AA* group, i.e., two normal structural alleles with high-expression promoter activity in position  $-221$  (*YA/YA*), one high-expression promoter and one low-expression promoter (*YA/XA*), or two low-

expression promoters (*XA/XA*); the *A/O* group, i.e., one variant structural allele (i.e., defective allele) and one normal structural allele combined with a high-expression promoter (*YA/O*) or a low-expression promoter (*XA/O*); and the *O/O* group, i.e., two defective structural alleles. The *X* allele is not carried on a haplotype containing structural *O* alleles.

Because *A/O* individuals carrying the low-expression *X* promoter allele on the functional *A* chromosome have very low MBL levels in their serum, we pooled these patients with those homozygous for two defective structural alleles (*O/O*) into an MBL-insufficient group and compared it with the rest of the patients (MBL-sufficient group) in some of the analyses.

**Detection of MBL binding to *P. falciparum*-infected human RBC by flow cytometry and immunofluorescence.** Purified human MBL from healthy donors for therapeutic use was obtained from the State Serum Institute, Copenhagen, Denmark (15) (a kind gift from Claus Koch). Five standard parasite isolates were used in this study: K1, FCR3, 3D7, 7G8, and DD2. Usually, assays were performed with four of these at convenience. In some assays, cryopreserved material from a previous study was used (41). The cryopreserved isolates were thawed and cultured as described previously (20). RBC infected by late developmental stages (hemozoin-containing trophozoites and schizonts) were purified (to  $>75\%$  parasitemia) from mycoplasma-free culture material by exposure to a strong magnetic field (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described (45). Aliquots of  $2 \times 10^5$  infected RBC, labeled with ethidium bromide (Sigma-Aldrich, St. Louis, Mo.) to allow flow cytometric exclusion of remaining uninfected RBC, were exposed to 0.5  $\mu\text{g}$  of purified MBL for 1, 2, 4, 6, 8, and 18 h to determine the capacity of MBL to bind to parasitized RBC and then sequentially incubated for 30 min with 0.25  $\mu\text{l}$  of mouse monoclonal MBL immunoglobulin G (IgG), 0.5  $\mu\text{l}$  of goat anti-mouse IgG (DAKO, Glostrup, Denmark), and 4  $\mu\text{l}$  of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG (DAKO). Samples were washed twice after each incubation step in HEPES buffer containing 25 mM HEPES, 155 mM NaCl, 5 mM  $\text{Ca}^{2+}$ , and 2% fetal calf serum. To inhibit MBL binding, increasing amounts of EDTA (2 to 10 mM) or mannose (5 to 25 mM) were added. Two-color flow cytometry data from 10,000 ethidium bromide-positive RBC were collected on a FACScan instrument (BD Biosciences, Franklin Lakes, N.J.). For quantification of FITC, the mean fluorescence intensity (MFI) was recorded. Nonspecific binding was evaluated by analysis of uninfected RBC kept under the same conditions as cultures of *P. falciparum* isolates.

Liquid-phase immunofluorescence was performed on cells treated as described above. Briefly an aliquot of the FACS sample was analyzed in a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany) under oil immersion at a total magnification of  $\times 1,000$ . Pictures were taken with a Leica DC100 camera and software.

**Characterization of the MBL-binding ligand from *P. falciparum*-infected RBC.** *P. falciparum* clone A4 was maintained in synchronous *in vitro* culture, and trophozoite stage-infected human RBC were enriched by gelatin flotation as previously described (3) (the material was kindly provided by Chris Newbold, Oxford, England). Approximately 60% of the RBC were infected. MBL was purified from human serum in accordance with a standard protocol (55) and incubated with aliquots of  $5 \times 10^7$  infected and uninfected RBC at 0 to 10  $\mu\text{g}$  of MBL per ml in 2.5 mM Veronal-145 mM NaCl-0.3 mM  $\text{MgCl}_2$ -1.5 mM  $\text{CaCl}_2$  for 30 min at  $37^{\circ}\text{C}$ . Cells were washed with  $4 \times 2$  ml of the same buffer and lysed in 0.1 M Tris containing 1% sodium dodecyl sulfate (SDS)-4 M urea-20 mM dithiothreitol, pH 8.0, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) with a 10% acrylamide gel. SDS-PAGE was carried out by the method of Laemmli with 3.0% (wt/vol) acrylamide stacking gels and 10% (wt/vol) acrylamide separating gels (31). Samples were prepared by adding, to an approximately equal volume, sample buffer consisting of 0.2 M Tris-HCl, 8 M urea, and 2% (wt/vol) SDS, pH 8.0, containing either dithiothreitol (at 20 mM unless otherwise stated) or 42 mM iodoacetamide, followed by incubation for either 30 min at  $37^{\circ}\text{C}$  or 5 min at  $90^{\circ}\text{C}$  (6). Molecular weight markers were run under the same conditions as the samples. Protein bands were visualized by Coomassie brilliant blue R-250 staining.

The gels were removed from the apparatus immediately after SDS-PAGE and rinsed with 48 mM Tris-39 mM glycine-0.0375% (wt/vol) SDS. Proteins were electroblotted onto Immobilon P membranes (Millipore) in an LKB 2117 Multiphor electrophoresis unit. The general procedures followed were those described by Towbin et al. (53). Electroblotting was carried out at 0.8 mA/cm<sup>2</sup> of gel area for about 2 h. Nonspecific binding sites on Immobilon P membranes were blocked after electroblotting by incubation of membranes for 1 h at room temperature or overnight at  $4^{\circ}\text{C}$  in PBS containing 0.1% (wt/vol) Tween 20 (PBS-Tween) and 25 mg of bovine serum albumin per ml. After electroblotting, the gels were stained with Coomassie brilliant blue to assess the extent of protein transfer. Strips from the Immobilon P membranes corresponding to the tracks on

TABLE 1. Frequencies of MBL genotypes in Ghanaians with and without malaria due to *P. falciparum*<sup>a</sup>

Genotype or parameter	No. (%) of persons					Total
	Hospital-based study			Community-based study		
	Cerebral malaria	Severe malarial anemia	Uncomplicated malaria	<i>P. falciparum</i> positive	<i>P. falciparum</i> negative	
<i>A/A</i>	67 (47.5)	30 (41.1)	56 (51.3)	65 (44.2)	39 (48.1)	257 (46.6)
<i>A/B</i>	1 (0.7)	1 (1.4)	0	0	1 (1.2)	3 (0.5)
<i>A/C</i>	58 (41.1)	33 (45.2)	44 (40.4)	66 (44.9)	33 (40.8)	234 (42.5)
Sum <i>A/O</i>	59 (41.8)	34 (46.6)	44 (40.4)	66 (44.9)	34 (42.0)	237 (43.0)
<i>B/C</i>	1 (0.7)	0	0	0	0	1 (0.2)
<i>C/C</i>	14 (9.9)	9 (12.3)	9 (8.3)	16 (10.8)	8 (9.9)	56 (10.2)
Sum <i>O/O</i>	15 (10.6)	9 (12.3)	9 (8.3)	16 (10.8)	8 (9.9)	57 (10.4)
Total	141 (100)	73 (100)	109 (100)	147 (100)	81 (100)	551 (100)
Frequency of <i>O</i> allele	0.32	0.36	0.28	0.33	0.31	0.32

<sup>a</sup> *A* is the common designation for the normal structural allele, while *O* indicates variant alleles. None of the groups deviated from the Hardy-Weinberg expectations ( $P > 0.5$ ). No statistically significant difference in gene frequencies was observed between the groups ( $P > 0.05$ ).

the gel were incubated with rabbit polyclonal anti-MBL antibodies in phosphate-buffered saline (PBS)-Tween for 2 h at room temperature (42). After being washed five times for 10 min (each time) with PBS-Tween 20, the membrane strips were incubated with goat anti-rabbit secondary antibodies conjugated to alkaline phosphates (Sigma, Poole, United Kingdom) for 2 h at room temperature following extensive washing with PBS-Tween. The blots were developed with the Sigma Fast 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (BCIP)-nitroblue tetrazolium substrate system.

RBC infected with *P. falciparum* were lysed with 5 ml of 10 mM sodium phosphatase buffer (pH 7.4) containing 1% NP-40, soyabean trypsin inhibitor (100  $\mu$ g/ml; Sigma), 5 mM iodoacetamide, 2 mM Pefabloc-SC (Boehringer Mannheim, Lewes, United Kingdom), and 50  $\mu$ g of pepstatin A (Sigma) per ml for 2 h at 4°C. Following centrifugation (Beckmann SW40Ti rotor) at 15,000 rpm for 30 min, the supernatant was loaded onto a fast protein liquid chromatography (FPLC) Mono Q column (Pharmacia, Uppsala, Sweden) pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.4). The column was washed extensively, and bound proteins were eluted with a linear gradient of 0 to 1.0 M NaCl in 10 mM sodium phosphate buffer (pH 7.4). The eluted fractions were tested for MBL-binding activity, and positive fractions were pooled. A control experiment with noninfected RBC was performed identically.

The pooled MBL-binding proteins were dialyzed against 10 mM sodium phosphate buffer (pH 7.4) and further fractionated on the FPLC Mono Q column. The MBL-binding fractions identified as described above (see Fig. 6) were analyzed by SDS-PAGE. The protein in the MBL-binding fractions was identified by N-terminal sequencing. Samples were subjected to SDS-PAGE and then electroblotted onto ProBlott membrane (Perkin-Elmer). This was stained with Coomassie brilliant blue, and the bands of interest were excised and sequenced with an Applied Biosystems 494A Procise sequencer. All solvents were from Perkin-Elmer.

A solid-phase adhesion assay was used to confirm that the eluate from the FPLC Mono Q column contained MBL-binding proteins. Aliquots of the column eluate were adsorbed onto microtiter plate wells (Nunc, Roskilde, Denmark) in 100 mM bicarbonate buffer (pH 9.6). Nonspecific binding sites were blocked with bovine serum albumin (3 mg/ml in 200  $\mu$ l of Tris-buffered saline). MBL (100  $\mu$ l; 2  $\mu$ g/ml), in the presence or absence of 50  $\mu$ g of soluble mannan (Sigma) per ml, was added to the coated wells in Tris-buffered saline containing 5 mM CaCl<sub>2</sub> and then washed, and horseradish peroxidase (HRP)-conjugated anti-MBL monoclonal antibodies (clone 131-1; State Serum Institute) were added. After extensive washing, the amount of bound MBL-anti-MBL-HRP complex was determined by adding the HRP substrate *o*-phenylenediamine dihydrochloride (Fast Enzyme System; Sigma). The reaction was stopped after 5 min with 3 M HCl, and the absorbance at 490 nm was measured.

**Statistical analyses.** Contingency table analyses and Fisher's exact test were used to compare frequencies. Kruskal-Wallis and Mann-Whitney tests were used to compare continuous data between carriers of the different MBL genotypes. Two-tailed tests were used throughout.

## RESULTS

**Disease association study.** Altogether, we investigated 551 Ghanaians for MBL variant alleles. In total, variant *O* alleles were found in 53.6% of the investigated individuals (allele frequency, 0.32). The predominant allele was the *C* allele, which was found in most individuals carrying variant alleles. The *B* allele was found with a frequency of less than 1%, while the *D* allele was missing.

In a comparison of the clinical groups in the hospital-based study, cerebral malaria, severe anemia, and uncomplicated malaria did not reveal any significant difference with regard to the distribution of MBL genotypes (Table 1) (three by three chi-square test,  $P = 0.7$ ). None of the groups deviated from the Hardy-Weinberg expectations. The same pattern was seen in the community-based study when those infected with malaria were compared with those negative for malaria by inspection of thick blood smears (two-by-three chi-square test,  $P = 0.84$ ).

When the low-expression promoter allele *X* was compared, the frequency was almost the same in each of the tested groups (Table 2). Because the low MBL expression *X* promoter allele produces very little MBL and is only found on an *A* background, we pooled this group with those with a homozygous defect for the structural alleles (*O/O*) into an MBL-insufficient group (*XA/O+O/O*) and compared it with the rest (*A/A+YA/O*). Again, no significant difference was observed between groups in the hospital- and community-based studies (Table 2).

We then tested whether the MBL genotypes could be associated with demographic or paraclinical data obtained on admission for patients with complicated malaria (Table 3). Homozygosity for variant alleles was significantly associated with increased *P. falciparum* parasitemia (Table 3 and Fig. 1) and low blood glucose levels (determined in fluoride plasma by the glucose oxidase method) (Table 3), but no significant association with age, duration of symptoms, hemoglobin level, or neutrophil count was found.

**Detection of MBL binding to *P. falciparum*-infected RBC.** Specific staining of *P. falciparum*-infected RBC by MBL in flow cytometry was observed (Fig. 2) for all five of the parasite

TABLE 2. Frequencies of low-MBL-producing promoter genotype *X* (−221) in Ghanaians with and without malaria due to *P. falciparum*<sup>a</sup>

Genotype or parameter	No. (%) of persons					Total
	Hospital-based study			Community-based study		
	Cerebral malaria	Severe malaria anemia	Uncomplicated malaria	<i>P. falciparum</i> positive	<i>P. falciparum</i> negative	
<i>YA/YA</i>	44 (31.2)	24 (32.9)	31 (28.4)	45 (30.6)	27 (33.3)	171 (31.0)
<i>YA/XA</i>	22 (15.6)	5 (6.5)	24 (22.0)	19 (12.9)	19 (13.6)	81 (14.7)
<i>XA/XA</i>	2 (1.4)	1 (1.4)	1 (0.9)	1 (0.7)	1 (1.2)	6 (1.1)
<i>YA/O</i>	50 (35.5)	25 (34.3)	35 (32.1)	52 (35.4)	27 (33.3)	189 (34.3)
<i>XA/O</i>	8 (5.6)	9 (12.3)	9 (8.3)	14 (9.5)	7 (8.6)	47 (8.6)
<i>O/O</i>	15 (10.6)	9 (12.3)	9 (8.3)	16 (10.8)	8 (9.9)	57 (10.4)
Frequency of <i>X</i> allele	0.12	0.11	0.16	0.12	0.12	0.13
Sufficient ( <i>A/A</i> + <i>YA/O</i> )	118 (83.7)	55 (75.3)	91 (83.5)	117 (79.6)	66 (81.5)	447 (81.1)
Insufficient ( <i>XA/O</i> + <i>O/O</i> )	23 (16.3)	18 (24.7)	18 (16.5)	30 (20.4)	5 (18.5)	104 (18.9)

<sup>a</sup> *A* is the common designation for the normal structural allele, while *O* indicates variant alleles. *Y/X* indicates a base substitution at position −221 (G to C). The *X* allele is associated with low MBL concentrations in serum. None of the groups deviated from the Hardy-Weinberg expectations ( $P > 0.5$ ). No statistically significant difference in gene frequencies was observed between the groups ( $P > 0.05$ ).

isolates used (Fig. 2). Variable binding of MBL to RBC was observed, depending on which parasite isolates were used (Fig. 3). The binding capacity reached a plateau after 4 h of incubation and was maintained throughout the incubation period (Fig. 3). As shown in Fig. 3, staining of uninfected RBC was also observed, but this staining was considerably weaker than when parasitized cells were used (Fig. 3). Inhibition of binding was observed when increasing amounts of EDTA or mannose were added at the MBL incubation step, resulting in dose-dependent inhibition (Fig. 4). Immunofluorescence microscopy showed that MBL bound distinctly to the outer surface of the RBC membrane (Fig. 5).

The same isolates showed different MBL staining in different experiments, which indicates that binding may vary with the stage of parasite development because purified parasite cultures may range from the mature trophozoite stage to the mature schizont stage.

**Characterization of the MBL ligand from *P. falciparum*-infected RBC.** MBL binding to *P. falciparum*-infected RBC was readily detectable by Western blotting of infected RBC lysates incubated with MBL. Uninfected RBC controls gave negligible binding, and no binding of MBL to infected RBC was seen in

the presence of EDTA. Because the experiments had to be done very rapidly and because of the variable loss of material due to the fragility of infected RBC, it was not possible to test saturation and binding kinetics; thus, the observed binding is a qualitative result rather than a quantitative one.

We then subjected infected cells to SDS-PAGE (reduced) and blotting onto an Immobilon P membrane. The membrane was incubated with purified MBL (5 µg/ml in 2.5 mM Veronal–145 mM NaCl–0.3 mM MgCl<sub>2</sub>–1.5 mM CaCl<sub>2</sub> for 1 h at room temperature) and washed in the same buffer, and bound MBL was detected with polyclonal anti-MBL and an alkaline phosphatase-conjugated second antibody. Appropriate controls were done with uninfected cells, and uninfected and infected cells were exposed to a first antibody but without MBL. A single strong band of about 80 kDa was obtained with infected cells plus MBL. This material was partially purified from detergent-solubilized membranes of lysed infected RBC by two Mono Q FPLC steps (Fig. 6). A major peak of Ca<sup>2+</sup>-dependent MBL binding centered on fraction 53 was observed. The binding to fractions 50 to 55 was inhibited in the presence of mannan (50 µg/ml). No material with similar chromatographic binding properties was found in uninfected RBC. SDS-PAGE

TABLE 3. Demographic and clinical characteristics on hospital admission of Ghanaians with complicated malaria due to *P. falciparum* stratified according to MBL genotypes<sup>a</sup>

Genotype or parameter	Age (yr)	Symptom duration (days)	Temp (°C)	Hemoglobin (mmol/liter)	Parasitemia (no. of organisms/µl)	Neutrophils (10 <sup>9</sup> /liter)	Glucose level <sup>d</sup> (nmol/liter)
<i>A/A</i>	4.0 (0.5–12.0)	1 (1–21)	37.9 (35.0–41.0)	6.5 (1.8–12.8)	82,069 (200–1,256,000)	6.2 (0.4–24.6)	6.7 (1–19.4)
<i>A/O</i>	3.5 (0.0–13.0)	3 (1–7)	38.0 (36.0–39.8)	5.9 (2.8–12.8)	57,824 (1,525–2,860,000)	8.2 (1.3–26.8)	6.2 (0.1–15.0)
<i>O/O</i>	3.7 (0.5–10)	3 (0–7)	38.0 (36.0–40.0)	6.2 (2.1–11.2)	15,765 (17640–1,038,000)	8.0 (2.7–40.6)	5.1 (0.8–8.2)
<i>P</i> value							
K-W <sup>b</sup>	0.6	1.0	0.9	0.5	0.03	0.1	0.02
m-w <sup>c</sup>	0.9	0.9	0.8	0.9	0.02	0.8	0.008

<sup>a</sup> *A* is the common designation for the normal structural allele, while *O* indicates variant alleles. Median values are indicated. Ranges are in parentheses.

<sup>b</sup> *P* values from Kruskal-Wallis test.

<sup>c</sup> *P* values from Mann-Whitney test (*A/A* + *A/O* versus *O/O*).

<sup>d</sup> Glucose levels were measured in fluoride plasma by the glucose oxidase method.

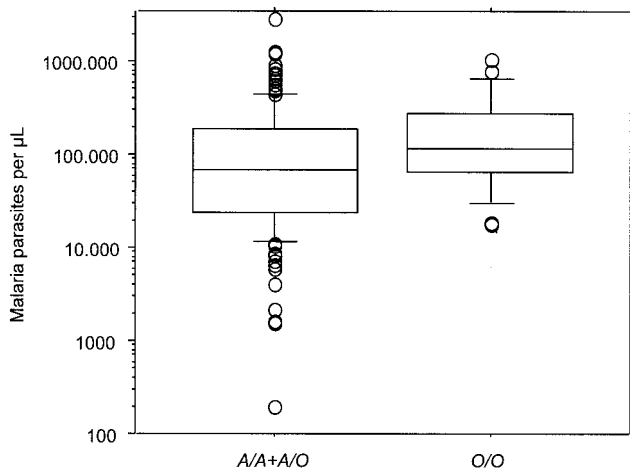


FIG. 1. Levels of *P. falciparum* parasitemia with respect to MBL genotypes. Shown are the levels of parasitemia due to *P. falciparum* on hospital admission and the MBL genotypes (A/A and A/O versus O/O;  $n = 188$  and 21, respectively) of patients with complicated malaria (cerebral malaria and severe anemia) (Mann-Whitney  $P = 0.02$ ).

analyses of fractions 52 to 54 revealed a single major band of 80 kDa. N-terminal sequencing of the 80-kDa band yielded a 16-amino-acid sequence identical to that reported for residues 22 to 37 of the cDNA-derived amino acid sequence of the 78-kDa glucose-regulated stress protein of *P. falciparum* (Table 4) (27).

**DISCUSSION**

Accumulating evidence indicates that MBL is a key molecule in the innate immune defense. In particular, genetic epidemiological studies have shown that the presence of the different variant alleles in the MBL gene is associated with an increased tendency for infections (8, 9, 47, 48, 49) but that they may be more important as prognostic markers in various infectious and autoimmune conditions (9, 10, 16, 21, 49). However, the view of a possible role for MBL in relation to malaria is, to some extent, contradictory (2, 33). In accordance with the findings of Bellamy and colleagues, who studied MBL polymorphisms in children with severe and mild malaria and con-

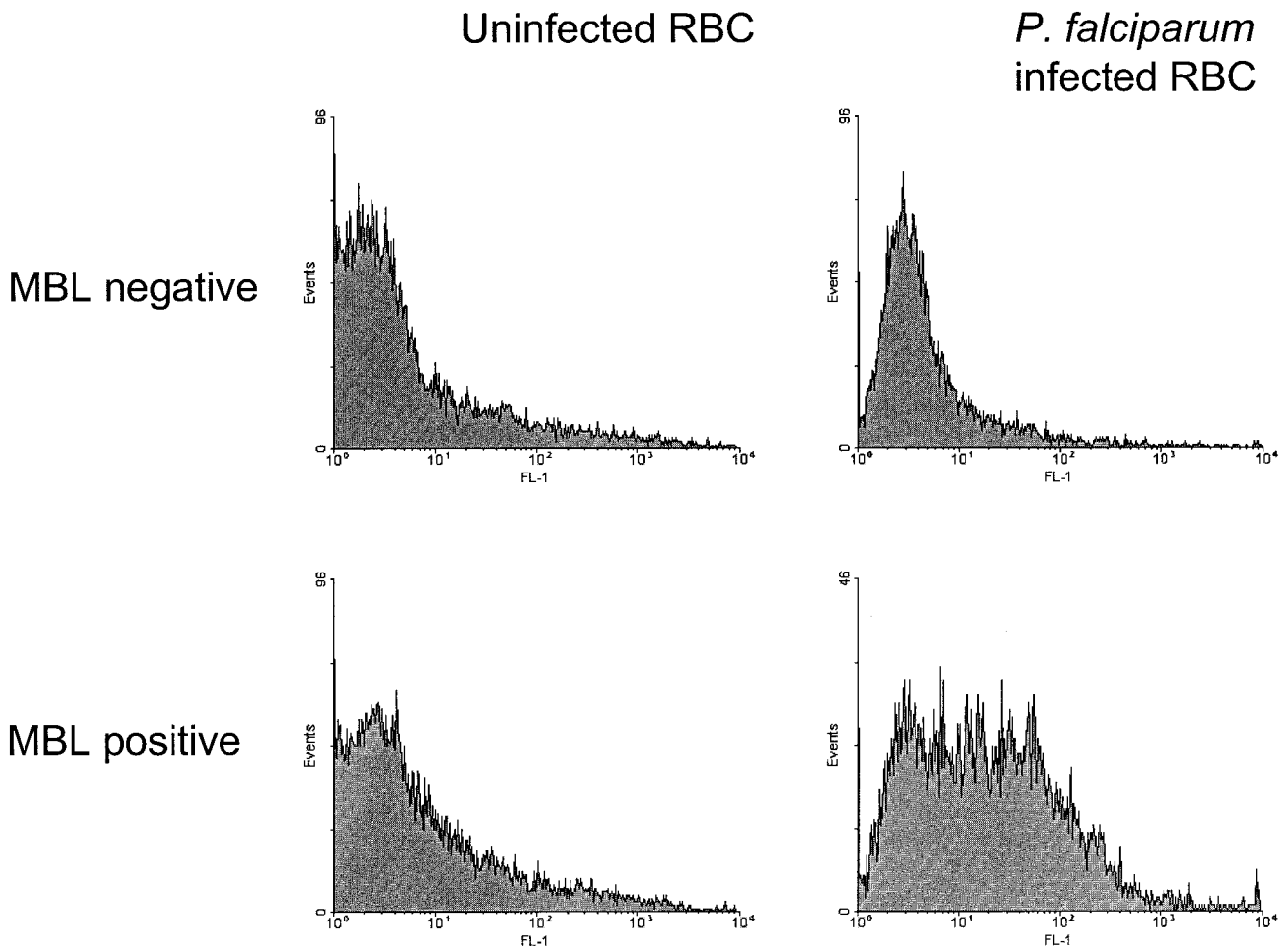


FIG. 2. Detection of MBL binding to *P. falciparum*-infected RBC by flow cytometry. Specific staining of *P. falciparum*-infected RBC by MBL after 18 h of incubation was observed by flow cytometry for all five of the parasite isolates used. Some staining of uninfected RBC was also observed, but this staining was weaker, ranging from 0 to 60% of the MFI of parasitized cells. Infected RBC, labeled by ethidium bromide to allow flow cytometric exclusion of remaining uninfected RBC, were exposed to 0.5 µg of purified MBL and then sequentially incubated with mouse monoclonal MBL IgG, goat anti-mouse IgG, and FITC-conjugated rabbit anti-goat IgG.

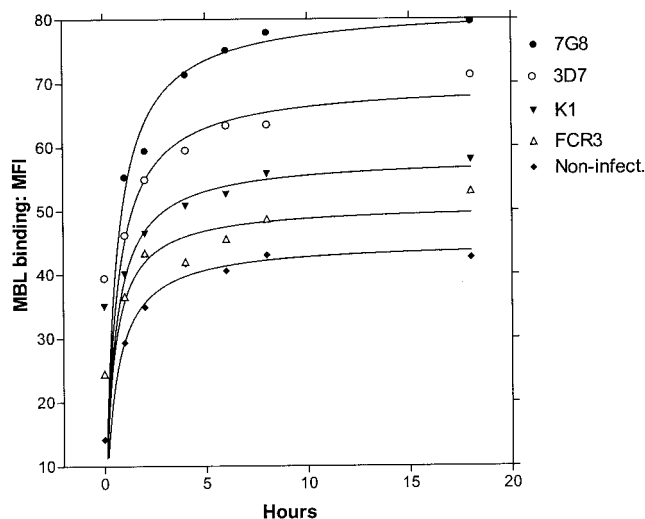


FIG. 3. Detection of MBL binding to *P. falciparum*-infected RBC by flow cytometry. Staining of *P. falciparum*-infected RBC by MBL detected by flow cytometry is presented as MFI and was dependent on the incubation time, reaching a plateau after 4 h. Different degrees of MBL binding were observed, depending on the parasite isolate used (7G8, 3D7, K1, or FCR3). Some staining of uninfected (Non-infect.) RBC was also observed, but this staining was weaker than that of parasitized cells. Infected RBC, labeled by ethidium bromide to allow flow cytometric exclusion of the remaining uninfected RBC, were exposed to 0.5  $\mu$ g of purified MBL and then sequentially incubated with mouse monoclonal MBL IgG, goat anti-mouse IgG, and FITC-conjugated rabbit anti-goat IgG.

trols without malaria in The Gambia, we found no significant difference between the frequencies of carriers with severe or mild malaria and controls (2). In Gambian children, the distribution of the variant alleles affecting the structural part of the MBL gene was almost identical to that which we observed in Ghana, with the *C* allele, as expected, being the predominant allele. In addition, we also tested for the promoter polymorphisms that have been shown to be associated with different MBL concentrations in serum (35). One polymorphism at position -221 (the *X* allele) upstream of exon 1 has been shown to have a particularly strong down regulating effect on the MBL concentration in serum. The *X* allele on a functional *A* chromosome, in combination with a structural *O* allele on

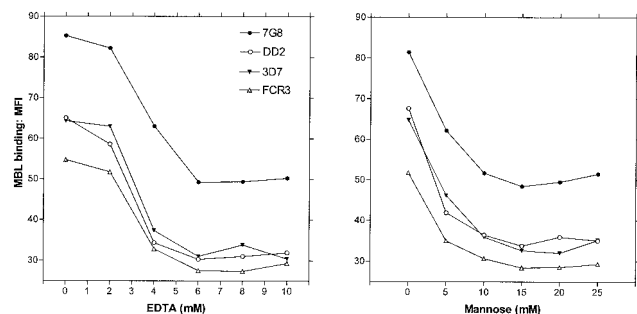


FIG. 4. Inhibition of MBL binding to *P. falciparum*-infected RBC by EDTA or mannose. The diagram illustrates inhibition of MBL binding to *P. falciparum* (isolate 7G8, DD2, 3D7, or FCR3)-infected RBC detected by flow cytometry when 0 to 10 mM EDTA or 0 to 25 mM mannose was added during the MBL incubation step, respectively.

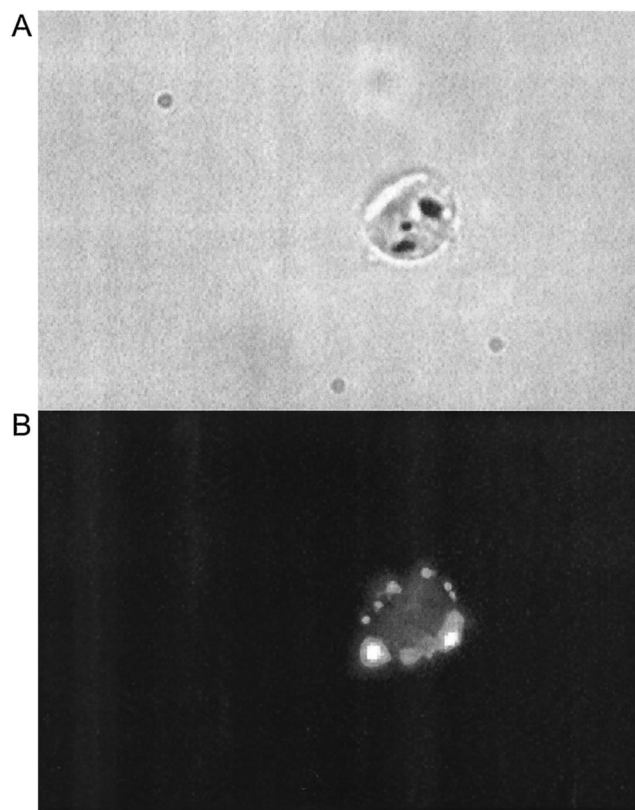


FIG. 5. Detection of MBL binding to *P. falciparum*-infected RBC by immunofluorescence microscopy. Panel A shows a liquid-phase light micrograph of an RBC infected with *P. falciparum* (original magnification,  $\times 1,000$ ). Panel B shows a corresponding immunofluorescence micrograph of MBL binding to *P. falciparum*-infected RBC. Only very weak binding to noninfected RBC was seen. MBL binding was visualized with the same antibodies as described in the legend to Fig. 2.

the other chromosome, has been shown to be associated with more progressive disease than the MBL *Y* allele at the same position in children with cystic fibrosis and in patients with rheumatoid arthritis (16, 21). Taking the *X* allele into account did not change our conclusion that MBL variant alleles and thus MBL deficiency do not predispose to an increased risk of *P. falciparum* malaria or that MBL deficiency may be associated with an increased risk of acquiring cerebral malaria or severe malarial anemia.

This conclusion opposes the conclusion in another association study addressing MBL polymorphisms and malaria from Gabon (33), which found that MBL polymorphisms were over-represented in a group of patients with severe malaria versus a group with mild malaria. However, the stratification criteria they used were different from those used in our study and the study of Bellamy et al. (2), which could explain this discrepancy. In agreement with the Gabon study, when we looked at defined parameters on admission that are known to be of pathophysiological importance in malaria, it was indeed revealed that homozygosity for MBL variant alleles (*O/O*) was associated with an increased *P. falciparum* parasitemic load and low glucose levels, compared with those of the MBL-competent counterparts (*A/A* and *A/O*). Even though there

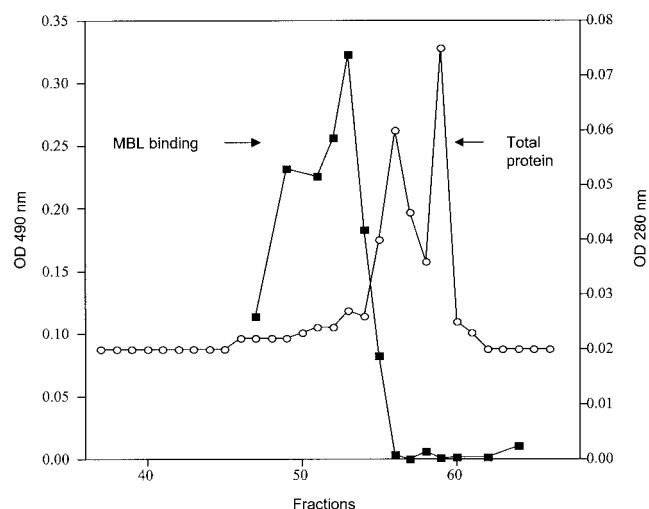


FIG. 6. Mono-Q FPLC of infected RBC lysate. Infected RBC were lysed and subjected to Mono-Q FPLC, and MBL-binding fractions were assayed as described in the text. Pooled MBL-binding fractions from the first chromatography were rerun on the same column at a lower protein loading level to increase resolution. The gradient portion of the chromatography is shown. Eluted proteins are shown by measurements of optical density (OD) at 280 nm. Corrected MBL binding is shown (binding of MBL in Ca<sup>2+</sup> ions after subtraction of the background binding in the presence of Ca<sup>2+</sup> ions and 50 μg of mannan per ml). The MBL-binding peak is associated with a minor peak in fraction 53.

was an overall inverse correlation between the glucose level and parasitemia, subanalysis showed that this inverse correlation was confined to the patients homozygous for the variant alleles (correlation coefficient, -0.6; *P* = 0.01), while no significant correlation was seen in *A/A* (correlation coefficient, -0.11; *P* = 0.33) or *A/O* (correlation coefficient, -0.14; *P* = 0.23) individuals. This finding could not be explained by a difference in the duration of disease and the hemoglobin level on hospital admission. Factors that are known to be associated with severe disease, namely, an increased neutrophil cell count and low hemoglobin levels, did not differ significantly between patients with the *A/A*, *A/O*, and *O/O* genotypes. However, in the present study, these parameters were not correlated with the level of *P. falciparum* parasitemia or blood glucose on admission. Thus, they may in part reflect pathophysiological mechanisms other than those that may implicate the MBL system. Taken together, the present findings indicate that MBL

to some degree may control the level of *P. falciparum* parasitemia. The mechanism by which MBL eventually may participate in the concerted action with many biological systems to combat malaria-causing parasites cannot be answered on the basis of this study. However, if hypoglycemia is caused by consumption of parasites in tissues, it is tempting to speculate that MBL may present malaria-infected cells to the phagocytic system and may thus be involved in the sequestration of the parasite. This could be mediated directly via MBL receptors or indirectly by the ability of MBL to enhance the deposition of complement opsonins (C3 and C4) (24).

These findings and the fact that MBL has recently been shown to bind to protein extracts of *P. falciparum* from RBC (25) prompted us to investigate whether MBL may function as an opsonin for RBC infected with *P. falciparum*, thus giving a possible molecular explanation for the present clinical findings. Distinct binding of MBL to RBC infected with *P. falciparum* could be observed when binding was assessed by flow cytometry. This binding was confined to the exterior side of the RBC membrane, as shown by immunofluorescence microscopy. Incubation of *P. falciparum*-infected RBC with either EDTA or mannose inhibited the binding of MBL in a dose-dependent fashion, indicating that the interaction was calcium dependent and mediated by the lectin domains in the globular heads of the MBL molecule. These results suggested that MBL bound to a *P. falciparum*-specific ligand exposed on the surface of the RBC after prolonged incubation. It is of particular interest that MBL revealed different abilities to bind to RBC containing different parasite isolates, indicating that MBL deficiency may be more or less important, depending on the carbohydrate structures exposed by the actual *P. falciparum* isolate. We attribute the MBL binding to noninfected RBC that we observed to the fact that the collagenous domain of MBL may interact with CR1 (CD35), a molecule expressed on RBC (19). It could be argued that the binding of MBL to RBC infected with *P. falciparum* was indirect because of an abnormality in the RBC membrane introduced by infection with *P. falciparum*. However, incubation of RBC with ionomycin, which flip flops and twists the RBC membrane, making the interior phospholipids accessible to annexin V on the exterior, did not expose any cryptic MBL ligand (A. J. Nauta et al., unpublished data).

We then tried to characterize a putative MBL ligand by purification and N-terminal sequencing of an MBL-binding protein from *P. falciparum*-infected RBC. This revealed a pro-

TABLE 4. Sequencing of an MBL-binding protein from RBC infected with *P. falciparum*<sup>a</sup>

Position	Amino acid at position:																													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	M	N	Q	I	R	P	Y	I	L	L	L	I	V	S	L	L	K	F	I	S	A	V	D	S	N	I	E	G	P	V
31	I	G	I	D	L	G	T	T	Y	S	C	V	G	V	F	K	N	G	R	V	E	I	L	N	N	E	L	G	N	R
61	I	T	P	S	Y	V	S	F	V	D	G	E	R	K	V	G	E	A	A	K	L	E	A	T	V	H	P	T	Q	T
631	V	K	L	Y	G	Q	P	G	G	P	S	P	Q	P	S	G	D	E	D	V	D	S	D	E	L					

<sup>a</sup> The protein in MBL-binding fractions of lysed *P. falciparum*-infected RBC was identified by N-terminal sequencing. The samples were run on SDS-PAGE and then electroblotted to ProBlott membranes (Perkin-Elmer). The blots were stained with Coomassie brilliant blue, and the bands of interest were excised and sequenced with an Applied Biosystems 494A Procise sequencer. The sequencing yielded a 16-amino-acid sequence identical to that reported for 78-kDa glucose-regulated stress protein of *P. falciparum* (accession number A48468; MUID 93133226) (27). The experimentally obtained sequence is amino acids 22 to 37. Only amino acids 1 to 90 and 631 to 657 are listed. Underlining indicates experimentally obtained sequence.

tein with a sequence identical to the cDNA-derived sequence of a *P. falciparum* 78-kDa glucose-regulated protein (grp78). grp78 is a homolog to a mammalian glucose-regulated stress protein (27). The cDNA-derived amino acid sequence contains two potential sites for attachment of N-linked carbohydrates, compatible with its interaction with MBL (27). The protein is expressed during the erythrocytic stage in both asexual and sexual parasites (28). Although the function of this protein is not fully understood, the expression of grp78 has been shown to increase in response to heat shock and is decreased after glucose deprivation in vitro (29). Thus, it is likely that grp78 is related to other heat shock proteins that may protect the parasite during fever episodes. We observe significant binding of MBL to RBC only after some hours of incubation, which could indicate that grp78 is translocated to the RBC membrane in situations in which the growth conditions are not optimal.

One challenging question in the field of MBL genetics has been why the variant alleles encoding low MBL concentrations in serum are so frequent in many ethnic groups, especially in areas in which malaria is endemic. The studies in The Gambia and Gabon and our study suggest that *P. falciparum* is not the selection factor that has caused the high frequency of variant alleles. On the contrary, our study and the study in Gabon suggest that MBL deficiency may increase the risk of *P. falciparum* hyperparasitemia and hypoglycemia; both are factors that can affect the outcome of the infection negatively. Thus, the maintenance of the *C* allele at high frequencies in most of sub-Saharan Africa implies that the putative negative influence the variant alleles may have on malaria is counterbalanced by some strong biological advantage (14). One hypothesis that has gained some support is that heterozygosity for an MBL variant allele, and thus a low MBL concentration, may hamper the uptake of intracellular bacteria and parasites to phagocytes, thereby hampering the replication of the pathogen (2, 13, 17, 23, 43). Another theory is that low levels of functional MBL may decrease excessive complement activation, which could be deleterious for the host (18, 32, 50). These two theories, however, are not mutually exclusive, and both could explain the high frequency of MBL alleles in many populations around the world. In this regard, it is of particular interest that both the populations in sub-Saharan Africa and the native populations of South America have extreme frequencies of different MBL variant alleles (alleles *C* and *B*, respectively) (36).

In conclusion, MBL deficiency did not, in this cross-sectional study design, seem to increase the risk of *P. falciparum* transmission or predispose to certain clinical subsets of malaria per se (cerebral malaria or anemia), but the observed data indicate that MBL is involved in regulating the degree of *P. falciparum* parasitemia and thus may be of prognostic importance because of the low blood glucose levels in the patients homozygous for the variant alleles. Moreover, we have shown that MBL may function as an opsonin for *P. falciparum* and identified *P. falciparum* glycoprotein grp78 as a putative ligand for MBL on infected RBC.

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