Complement Factor D, Albumin, and Immunoglobulin G Anti-Band 3 Protein Antibodies Mimic Serum in Promoting Rosetting of Malaria-Infected Red Blood Cells[∇]

Alexander Luginbühl,¹ Milica Nikolic,^{1†} Hans Peter Beck,² Mats Wahlgren,³ and Hans U. Lutz^{1*}

ETH Zurich, Institute of Biochemistry, Schafmattstrasse 18, CH 8093 Zurich, Switzerland¹; Swiss Tropical Institute, Socinstrasse 57, CH 4002 Basel, Switzerland²; and Karolinska Institute, Microbiology and Tumor Center, Stockholm, Sweden³

Received 20 September 2006/Returned for modification 24 October 2006/Accepted 21 January 2007

Rosetting of *Plasmodium falciparum*-infected red blood cells (parasitized RBC [pRBC]) with uninfected RBC has been associated in many studies with malaria morbidity and is one form of cytoadherence observed with malarial parasites. Rosetting is serum dependent for many isolates of *P. falciparum*, including the strains FCR3S1.2 and Malayan Camp studied here. We identified the three naturally occurring components of sera which confer rosetting. Complement factor D alone induced 30 to 40% of de novo rosetting. Its effect was additive to that of 0.5 mg/ml albumin and to that of 15 ng/ml of naturally occurring antibodies to the anion transport protein, band 3. The three components together mediated rosetting as effectively as 10% serum. De novo rosetting experiments showed that naturally occurring anti-band 3 antibodies as well as factor D were effective only when added to pRBC. Factor D appeared to cleave a small fraction of a protein expressed on the surface of pRBC.

Cerebral malaria occurs from infections with *Plasmodium falciparum*, the parasite species that shows sequestration in the periphery. Sequestration is a means by which parasitized red blood cells (pRBC) escape from their clearance from the reticuloendothelial system. It is mediated by cytoadherence and rosetting of pRBC with uninfected RBC. Clinical studies and autopsies have indeed revealed rosettes in blood vessels from malaria victims (19, 20, 40, 43), and parasites isolated from patients with severe disease formed more and larger rosettes than those from uncomplicated cases (4, 47). For many parasite isolates, in vitro rosetting is dependent on serum factors that are also present in nonimmune, healthy persons. Thus, it is more than a coincidence that the mortality from cerebral malaria is highest in very young children, who have not developed immunity against the parasite but have partially established their innate immune systems. The serum factors that have been shown to mediate rosetting are immunoglobulins (15, 43, 45), albumin (48), and a third so far unidentified small component which does not bind to concanavalin A (45). We embarked on a study of these serum factors in more detail because neither the specificities of the involved innate immunoglobulins (naturally occurring antibodies [NAb]) nor the role of complement has been investigated.

MATERIALS AND METHODS

Parasite cultures. The *P. falciparum* strains FCR3S1.2 and Malayan Camp (MR4; ATCC, Manassas, VA) were cultured according to standard procedures (46). Parasites were cultivated with 5% washed RBC (blood type O Rh⁺; Swiss

Red Cross, Zurich, Switzerland) in malaria culture medium (MCM; 10.43 g/liter RPMI 1640, 6 g/liter HEPES, 2 g/liter NaHCO₃, 0.1% neomycin, 0.05 g/liter hypoxanthine) supplemented with 10% heat-inactivated (30 min, 56°C) type AB Rh⁺ serum (complete MCM). When the parasitemia reached 5 to 10%, the culture was diluted to achieve 0.2 to 0.5% parasitemia and culture was continued. Twice a month, cultured parasites were enriched for the rosetting phenotype (49).

Rosette disruption. A rosetting parasite culture containing *P. falciparum* late stages at a parasitemia of 5 to 10% was centrifuged for 5 min at 500 × g. Cells were washed twice with phosphate-buffered saline (PBS)–glucose (pH 7.4) and resuspended to a 20% hematocrit in PBS–glucose containing 5 mM sodium citrate, and rosettes were disrupted by passing the resuspended cells 20 times through a 23-gauge 0.6-mm-diameter needle. Cells were then washed once with PBS–glucose containing 5 mM sodium citrate and twice with PBS–glucose and then used for rosette reformation assays or for pRBC enrichment.

Enrichment of pRBC bearing *P. falciparum* late stages. A magnetic cell sorting (MACS CS) column (Miltenyi Biotec, Bergisch Gladbach, Germany) was placed into the magnetic field (VarioMACS; Miltenyi Biotec), blocked with PBS–glucose containing 0.5% human serum albumin to minimize unspecific cell binding, and equilibrated in PBS–glucose as described elsewhere (50). The column containing ferromagnetic fibers will enrich infected RBC while in the magnetic field because of the high content of hemozoin in the RBC (37). Twenty-five milliliters of a cell suspension after rosette disruption was loaded at 5% hct on the column at a flow rate of 3.5 ml/min. The column was washed with PBS–glucose and removed from the magnetic field, and bound cells were eluted with PBS–glucose. Enriched pRBC (>95% pRBC bearing late-stage parasites) were used on the same day for de novo rosette formation or were surface radioiodinated.

Rosette reformation. Rosette reformation was studied with cell suspensions after rosette disruption. Pelleted cells were resuspended in MCM to 10% hct. This cell suspension was made 5% hct with MCM containing purified human serum proteins or human serum at the concentrations indicated below (see Fig. 1). In addition, a negative control (no protein/serum addition) and a positive control (addition of 10% heat-inactivated AB Rh⁺ serum) were prepared. These mixtures were incubated with shaking for 45 min at room temperature (RT). Aliquots (30 μl) of these cell suspensions were mixed with 4 μl of a 10-μg/ml acridine orange solution to stain nuclei for visualization. The extent of rosetting was determined for at least 200 pRBC as the number of pRBC that bound two or more uninfected RBC. The relative extent of rosetting is given in the figures and was calculated from the extent of rosetting as a percentage of that found with the positive control. To illustrate that rosette reformation reached values similar to those in the original cultures, the extent of rosetting in the controls is indicated as a percentage of that determined for the original cultures. Rosetting was read

* Corresponding author. Mailing address: ETH Zurich, Institute of Biochemistry, HPM D14, Schafmattstr. 18, CH 8093 Zurich, Switzerland. Phone: 41 44 632 3009. Fax: 41 44 632 1269. E-mail: hlutz@bc.biol.ethz.ch.

† Present address: Institute of Pharmaceutical Sciences, ETH Zurich, Zurich, Switzerland.

[∇] Published ahead of print on 29 January 2007.

by one investigator (A.L.), who also performed the rosetting assays. Hence, the readings were not done blinded. Instead, another investigator who was not involved in setting up the rosetting experiments and did not know the conditions (M.N.) read some of these rosetting slides independently. Her data deviated by not more than ± 3 to 4% of those determined by A.L.

De novo rosette formation. De novo rosette formation was studied with enriched pRBC and fresh, uninfected RBC. Washed RBC (type O Rh⁺) and enriched pRBC were washed twice with PBS-glucose and once with MCM and resuspended in MCM to 50% hct. RBC and pRBC were mixed to achieve 5% hct and 7% parasitemia and were supplemented with the concentrations of purified serum proteins indicated below (see Fig. 2). The mixtures of RBC, pRBC, and proteins/sera were incubated and the extent of rosetting was determined as outlined below. In some experiments, fresh, uninfected RBC and pRBC were preincubated with purified human serum proteins. Cells were then washed once with an excess of MCM to remove unbound protein. DFP-treated type AB Rh⁺ serum (native serum supplemented with 0.1% diisopropylfluorophosphate [DFP; Fluka, Buchs, Switzerland]) and RBC or pRBC were added to the preincubated cells, and the extent of rosetting was determined.

Surface ¹²⁵I labeling of pRBC and RBC. Surface ¹²⁵I labeling of pRBC and RBC was performed for 45 min at RT with 0.5 mCi Na¹²⁵I (0.5 mCi; Amersham, Little Chalfont, England) on 1 ml of pRBC/RBC at 20% hct by the lactoperoxidase method (38). Radioiodinated cells were washed twice with 1 ml of stopping buffer and twice with PBS-glucose. Finally, ¹²⁵I-labeled pRBC and RBC were used for incubation with factor D, and membranes of these cells were isolated.

Effect of factor D on pRBC and RBC. A portion of complement factor D (gift from J. Schifferli, University Hospital, Basel, Switzerland) was inactivated by adding 2 μ l of DFP to 48 μ l of factor D solution (0.15 mg/ml factor D, 75 mM NaH₂PO₄, 100 mM NaCl, pH 7.4). ¹²⁵I-labeled or unlabeled pRBC and RBC were incubated at a 15% hematocrit (in MCM) under shaking with 2 μ g/ml of either active or DFP-inactivated factor D for 45 min at RT. Thereafter, cells were centrifuged for 5 min at 500 $\times$ g, and the supernatant was recovered. Membranes of the incubated cells were isolated as described below (see Fig. 3), and parasite proteins in the supernatants were complexed with a hyperimmune serum pool (2%) from asymptomatic malaria patients from Papua New Guinea. The mixtures were incubated for 1 h at 37°C and subsequently kept on ice for 30 min. A fraction of this material was added to immobilized protein G, and the mixture was incubated overnight at 4°C. The protein G beads were washed, and bound protein was eluted twice with 1% SDS–5 mM *N*-ethylmaleimide.

Isolation of RBC membranes. Whole human blood (type A Rh⁺) depleted of leukocytes (Swiss Red Cross, Zurich, Switzerland) was centrifuged, and packed RBC were resuspended with PBS-glucose (10 mM NaH₂PO₄, 150 mM NaCl, 1 g/liter D-glucose, pH 7.4) supplemented with 1 mM DFP (Fluka, Buchs, Switzerland). The cells were then pelleted and washed three times with PBS-glucose, and membranes were prepared as outlined previously (39). RBC membranes were either used for band 3 protein purification or solubilized and alkylated by adding sodium dodecyl sulfate and *N*-ethylmaleimide to final concentrations of 1% and 5 mM, respectively. Aliquots were stored at –70°C.

Purification of band 3 protein. Band 3 protein was purified from Triton X-100 extracts of RBC membranes using anion-exchange and affinity chromatographies (26) as described previously (29). Purified band 3 protein and commercially available human serum albumin were coupled to Affigel-15 as described previously (29). Band 3 protein was immobilized by Schiff base chemistry to Chemobond plates (Dr. Ernst Fischer Laboratories, Dübendorf, Switzerland) for enzyme-linked immunosorbent assay (ELISA) (33).

Purification of IgG anti-band 3 and IgG anti-albumin NAb. Naturally occurring anti-band 3 (29, 31) and anti-albumin antibodies (35) were purified from pooled, whole human IgG (Sandoglobulin; ZLB Behring, Berne, Switzerland) after removal of antispectrin NAb (34). A portion of purified anti-band 3 NAb (1.2 mg) was repurified by a batch procedure following treatment with 5 M urea for 1 h at RT at 0.4 mg/ml to dissociate preexisting NAb complexes (23). The repurified material bound significantly less to intact IgG and to its light and heavy chains on immunoblots (not shown).

SDS-polyacrylamide gel electrophoresis and immunoblots. SDS-polyacrylamide gel electrophoresis was performed as outlined previously (11). Samples were reduced for 3 min in a boiling water bath, alkylated, and run at 8% total acrylamide. Either the gels were stained, dried, and exposed to PhosphorImager screens (Molecular Dynamics) for autoradiography or the polypeptides were blotted onto Immobilon P (Millipore, Bedford, MA). Blots were incubated with 0.5% rabbit antiserum against an acidic terminal segment of PfEMP1 (14) and then with ¹²⁵I-labeled protein G as outlined previously (22).

Monomeric IgG was isolated from Sandoglobulin (ZLB Behring, Berne, Switzerland) by gel filtration in PBS (pH 7.4) on a Sephacryl S300 column (2.5 by 80 cm; Amersham, Little Chalfont, England).

FIG. 1. Rosette reformation induced by native, heat-inactivated, and DFP-treated serum supplemented with or without either active or inactivated complement factor D. (A) Disrupted and washed cells from cultures were incubated with either heat-inactivated (black line), DFP-treated (dashed line), or native AB Rh⁺ serum (gray line) from the same donor, and the relative extents of rosetting were determined. The control (100%) with heat-inactivated serum showed 84.8% of the rosetting extent of the original culture. Data points show the average relative rosetting $\pm$ standard deviation for at least three independent experiments. (B) Cells were treated as described for panel A, but resuspended cells were also incubated with increasing concentrations of DFP-treated serum to which 2 μ g/ml of active or DFP-inactivated complement factor D was added. The relative extents of rosetting are shown for native serum (gray line), DFP-treated serum (dashed black line), DFP-treated serum supplemented with active factor D (black line), and DFP-treated serum containing inactivated factor D (dashed gray line). conc., concentration.

ELISA. Anti-band 3 NAb concentrations in sera were determined by an ELISA using band 3 covalently bound to Chemobond plates (33). Sera were diluted 1:400 with 10 mM NaH₂PO₄, 150 mM NaCl, 10 mM EGTA, 50 mg/ml albumin, and 0.04% Triton X-100 (pH 7.4) and incubated overnight in triplicate on immobilized band 3. The wells were washed, and bound IgG molecules were revealed as outlined elsewhere (23).

RESULTS

Previous studies on the rosetting of pRBC with uninfected RBC were carried out with either native or heat-inactivated sera (41, 45, 48). Both types of sera added at 3 to 10% mediated rosette reformation to the extent found in culture. In contrast to this, serum that was pretreated with DFP (DFP-treated serum) (12) reformed only 70 to 80% of the rosettes formed with native or heat-inactivated serum (Fig. 1). While the DFP treatment of sera inactivates complement, heat inactivation not only destroys the activity of factor D (1) but also aggregates immunoglobulins, a phenomenon known to yield, e.g., higher ELISA readings for IgG antibodies in sera (8, 18, 53). In DFP-treated serum, no protein aggregation is induced. DFP chemically modifies a serine residue in the active site of complement factor D, a constitutively active serine protease of the alternative pathway of the complement system (12, 25). The addition of active, but not inactivated, factor D (2 μ g/ml) to increasing concentrations of DFP-treated serum fully restored rosette reformation to the extent obtained by 10% native human serum (Fig. 1 B). The effect of factor D was the same irrespective of whether it was further supplemented with

FIG. 2. De novo rosette formation induced by DFP-treated serum with either active or inactivated complement factor D. Rosettes from cultures were mechanically disrupted, and RBC infected with late-stage parasites were enriched on MACS columns. Enriched pRBC (parasitized RBC) or freshly washed, uninfected RBC were then preincubated with either 2 μ g/ml of active or DFP-inactivated complement factor D. Upon preincubation and a single washing step, the missing uninfected or infected RBC, respectively, and 3% DFP-treated AB Rh⁺ serum were added, the suspensions were incubated, and the relative extents of rosetting were determined (the control with heat-inactivated serum showed 76% of the rosetting found in the original culture). Black and gray bars show the extents of de novo rosette formation as mediated by 3% DFP-treated serum with active (black bars) or inactivated (gray bars) complement factor D. As additional controls, enriched pRBC were incubated in 3% DFP-treated serum (diagonally hatched bar) and 3% native serum (horizontally hatched bar). Mean values + standard deviations from four independent experiments are given.

factor B (not shown). Interestingly, active complement factor D alone, at a concentration of 2 μ g/ml, induced rosette reformation by 30% in the absence of any other serum protein (Fig. 1B). Complement factor D induced not only reformation of rosettes but also de novo rosette formation (Fig. 2). It stimulated de novo rosetting by 20 to 30%. Moreover, de novo rosette formation required that complement factor D interact with infected RBC rather than with fresh uninfected RBC (Fig. 2).

Factor D had an additive effect on rosetting along with other known rosetting factors. As little as 0.5 mg/ml human serum albumin was equally effective as 5 mg/ml in mediating 40% rosette reformation (Fig. 3A), in part by restoring the discoid shape of washed, echinocytic RBC (not shown). Human serum albumin and complement factor D together at their optimal concentrations induced rosette reformation to a level of 70 to 80% of the maximal rosetting (Fig. 3B). Thus, the effects of albumin and complement factor D were additive. Since whole human immunoglobulin stimulates rosetting (45, 48), we anticipated the involvement of NABs that have tissue homeostatic roles in the clearance of oxidatively stressed (27) and senescent (28) RBC and have been shown to bind to malaria-infected RBC (52). Monomeric, whole human IgG increased the level of rosette reformation by 25 to 30% at 1.5 mg/ml in 0.5% DFP-treated serum (Fig. 4A). IgG anti-band 3 NAB and repurified IgG anti-band 3 NAB preparations induced the same increases in rosette reformation, but at concentrations of 0.15 μ g/ml and 5 to 15 ng/ml, respectively. IgG antialbumin NABs (35) had no effect on rosette reformation at concentrations up to 0.5 μ g/ml. Repurified IgG anti-band 3 NABs also increased de novo rosette formation by 25% when added to enriched pRBC at 50 ng/ml prior to rosette formation in 1% DFP-treated serum (Fig. 4B). On the other hand, addition of the same concentration of anti-band 3 NABs to fresh, uninfected

FIG. 3. Effect of complement factor D and/or human serum albumin on rosette reformation. (A) After disruption of rosettes, cells were washed and then incubated with increasing concentrations of human serum albumin with or without 0.5% DFP-treated AB Rh⁺ serum. The addition of 0.5% serum increased the total albumin concentration by 0.3 mg/ml. Reforming rosettes were counted, and the relative extents of rosetting were calculated using heat-inactivated serum, which showed 93% of the rosetting determined in the original culture, as a reference. The relative extents of rosetting for albumin alone (gray line) and for albumin supplemented with 0.5% DFP-treated serum (black line) are shown. For all data points, the average relative extent of rosetting $\pm$ standard deviation for at least three independent experiments is given. (B) Following rosette disruption, the effect of complement factor D (2 μ g/ml; filled bars) and of the MCM (hatched bars) on rosette reformation was studied either with or without 0.5 mg/ml human serum albumin. Mean values + standard deviations for three independent experiments are shown.

RBC had no effect on rosette formation. Pooled human IgG incubated with uninfected and parasitized RBC at the corresponding concentrations did not increase rosette formation (Fig. 4B).

We then studied the effect of these proteins on de novo rosette formation in the absence of serum (Fig. 5A). Human serum albumin, complement factor D, and repurified anti-band 3 NABs were incubated either individually or in combination with uninfected RBC and enriched pRBC bearing parasite late stages of strain FCR3S1.2, and the extent of their de novo rosette formation was assessed. Albumin, complement factor D, and anti-band 3 NABs induced rosette formation to a level of 15 to 25% when tested individually, with factor D being the most efficient rosetting mediator among the three proteins. The combinations of two of the three proteins exerted more than an additive effect. Finally, a combination of all three proteins induced 97% de novo rosetting. Similar results (100% de novo rosetting) were obtained with RBC infected with parasites of a different strain, the Malayan Camp strain (Fig. 5B). Thus, the three serum proteins at 0.5 mg/ml albumin, 2 μ g/ml factor D, and 15 ng/ml anti-band 3 NABs could fully mimic the effect of 10% native serum in inducing de novo rosette formation on cells infected with two different *P. falciparum* strains (FCR3S1.2 and Malayan Camp) which are both well-characterized rosetting strains (2, 5, 9, 13, 48). The rela-

FIG. 4. Effect of IgG anti-band 3 NABs, IgG anti-albumin NABs, and whole human IgG on rosette reformation (A) and de novo rosette formation (B). (A) Following rosette disruption and washing, rosette reformation was determined (means $\pm$ standard deviations) with cells incubated with increasing concentrations of IgG anti-band 3 (dashed black line), repurified IgG anti-band 3 (solid gray line), anti-albumin NABs (dashed gray line), or monomeric, whole human IgG (solid black line) in the presence of 0.5% DFP-treated AB Rh⁺ serum. The relative extent of rosetting is shown as a percentage of the rosetting of the control, which revealed 98% of the rosetting found in the original cultures. (B) Infected RBC (parasitized RBC) and freshly washed uninfected RBC enriched on MACS columns were preincubated with 0.05 $\mu\text{g/ml}$ repurified IgG anti-band 3 NABs (black bars) or monomeric, whole human IgG (gray bars). Upon preincubation and a single washing step, the missing uninfected or infected RBC and 1% DFP-treated serum were added, the cells were incubated, and the extents of rosetting were determined. The negative control comprised 1% DFP-treated serum (hatched bar). conc., concentration.

tive extents of de novo rosette formation induced by one or two components differed between the strains, but the requirement for all three rosetting factors for maximal rosetting was consistent. We do not know whether these differences originate from antigenic variants or emerged from the fact that de novo rosette formation with pRBC of the Malayan Camp strain was studied exclusively at the protein concentrations optimized for the FCR3S1.2 strain.

While IgG anti-band 3 NABs had a significant effect on malaria rosetting as shown here, the titers of these NABs do not appear to correlate with those of severe malaria (21). We addressed this discrepancy by studying IgG anti-band 3 NAB titers on serum samples available from a field study (16) with children from Papua New Guinea. Children in the high-infection group had significantly higher anti-band 3 NAB titers than those in the low-infection group ($P < 0.0018$), with up to threefold-higher titers (Fig. 6).

Since active complement factor D increased rosetting only by interacting with pRBC, we studied whether it modified surface-iodinated pRBC. These preliminary studies did not reveal apparent differences in the band patterns of ¹²⁵I-labeled

FIG. 5. De novo rosette formation induced by combining albumin, IgG anti-band 3 NABs, and complement factor D with pRBC infected with strain FCR3S1.2 (A) or Malayan Camp (B). pRBC infected with late stages from *P. falciparum* strain FCR3S1-2 (A) or Malayan Camp (B) were enriched on MACS columns. Their relative extents of rosetting were determined upon incubation with uninfected RBC in the presence of either a single protein, the given combinations of two proteins, or all three proteins at the following concentrations: 0.5 mg/ml human serum albumin, 0.015 $\mu\text{g/ml}$ anti-band 3 IgG, or 2 $\mu\text{g/ml}$ active complement factor D. HSA, human serum albumin; D, factor D; Anti-B3, anti-band 3. Mean values of the relative extents of rosetting $\pm$ standard deviations were calculated from three independent experiments against controls with 10% heat-inactivated serum, which revealed 85% of the extent of rosetting in the original cultures. Note, however, that the extents of rosetting in cultures of the two strains differed, with 73% for FCR3S1.2 and 39.3% for the Malayan Camp.

membranes, whether incubated with active or DFP-inactivated factor D. On the other hand, eluates from protein G beads loaded with supernatants from pRBC which had been incubated with a serum pool from hyperimmune malaria patients revealed one polypeptide of about 65 kDa which was seen primarily in eluates from factor D-treated pRBC. These and analogous preliminary results suggest that factor D may cleave a small fraction of a protein from pRBC, but not from RBC.

DISCUSSION

The serum components and the processes which enable rosette formation are poorly understood. This is due in part to the fact that the majority of in vitro studies have been carried out with heat-inactivated serum in which viruses and complement are inactivated but in which nonphysiologic protein-protein interactions increased, as exemplified by ELISA measurements on several types of IgG antibodies (8, 18, 53). ELISA readings with samples from heat-treated serum are significantly increased because a specific IgG antibody will no longer bind on its own but will bind as a heat-induced artificial IgG complex that subsequently captures higher numbers of secondary antibodies. Hence, it is likely that aggregation of IgG NABs

FIG. 6. IgG anti-band 3 NAb titers in Tanzanian children with a high or low risk for clinical malaria. IgG anti-band 3 NAb titers were determined by ELISA in the sera of Tanzanian children (<2.5 years) with apparently asymptomatic malaria. Children with parasite densities of <500/ μ l at any one time point throughout the study were grouped into the "low-infection group." If they presented frequently high parasite rates, they were grouped in the "high-infection group." Instead of using peptides from band 3 protein, we performed ELISAs on covalently bound intact band 3 protein in the presence of a nonionic detergent (33). The anti-band 3 titers in the two risk groups of children are shown as mean values $\pm$ standard deviations from three independent determinations at one time point for each patient. The range of anti-band 3 NAb concentrations for healthy controls (Swiss children of <2.5 years) is indicated by horizontal lines with optical density values at 405 nm (OD405) of 0.105 ± 0.051 ($n = 25$). The different symbols illustrate the children's hemoglobin levels in grams per liter as follows: closed diamonds, >100; open diamonds, 90 to 99; open triangles, 80 to 89; open circles, 70 to 79; closed circles, <69. Preliminary data from longitudinally taken samples from five of these children showed that at times of an upcoming infection, a significant loss of hemoglobin occurred and a 30% increase of anti-band 3 titers followed within a month. Hence, anti-band 3 NAb concentrations appear to change as a consequence of the number of infections and extent of hemolysis, but to address this longitudinally, field studies with children are required.

in serum augments their effect such that the stimulatory effect of IgG NAb on rosetting may be significantly more pronounced than in native serum and thereby compensates for the lack of factor D. Thus, native and heat-inactivated sera were equally effective in mediating rosetting, as shown here and earlier (45), but not necessarily by the same mechanisms. Here we show that serum in which serine proteases were inactivated by DFP could restore rosette formation only to 70 to 80% of the extent found with native serum. The inhibitory effect of DFP could not be compensated for by adding up to 10% of DFP-treated serum which contained saturating concentrations of anti-band 3 NAb and albumin, as shown in the rosetting experiments illustrated in Fig. 2A and 4A. However, reconstitution with physiological concentrations of factor D overcame the inhibition by DFP. Factor D had to be present with pRBC rather than uninfected RBC to induce rosetting. Factor D appeared to cleave a small fraction of a protein expressed on pRBC. Thus, factor D is one of the components needed for physiologic rosetting. This observation calls for a detailed investigation to understand whether the process involves a parasitic protein with a high diversity (3, 6, 44), which might influence the cleavability and the extent to which a certain isolate undergoes serum-dependent rosetting.

We have identified factor D as a so far unknown mediator of pRBC rosetting. Factor D, as a 25-kDa nonglycosylated serum protein with a pI of 6.6 to 7.0 (10), may represent the missing

low-molecular-weight serum factor in rosetting that does not bind to concanavalin A but that is important for rosetting (45). Factor D (2 μ g/ml) alone induced 30 to 40% of rosetting, but its effect was additive to that of 0.5 mg/ml human serum albumin and to 15 ng/ml anti-band 3 NAb directed to the anion transport protein. This anti-band 3 NAb concentration is well within the range of these NAb in human serum, which represent 1.5×10^{-5} of all human IgG molecules (29). Anti-band 3 NAb exerted their rosette-inducing property when added to pRBC but not when added to fresh uninfected RBC. Thus, they may have bound to oligomerized band 3 protein, which is far more abundant on pRBC than on senescent normal RBC (17). In contrast, antialbumin NAb were ineffective at concentrations up to 500 ng/ml. Thus, albumin, factor D, and anti-band 3 NAb fully replaced 10% native serum in mediating rosetting. Although parasite cultures and rosetting assays were carried out with 10% serum or heat-inactivated serum, we think that the three components have the same rosette-promoting effects in full-strength serum. The reasons are that (i) the extent of rosetting increases by only a few percent when assays are carried out in full-strength rather than 10% serum (48) and (ii) anti-band 3 NAb exert their rosette-promoting effect already at 15 ng/ml, a concentration that is lower than that in 10% serum (22 ng/ml). Since anti-band 3 NAb and factor D are part of the innate immune system and have physiological roles in healthy humans, prevention of rosetting cannot be achieved by inhibiting factor D or by interfering with anti-band 3 NAb.

The ability of anti-band 3 NAb to promote de novo rosetting when they are added to pRBC is unique but does not explain how pRBC-bound IgG anti-band 3 NAb may form a bridge to uninfected RBC. Since anti-band 3 NAb depleted of anti-IgG reactive NAb were most effective, it is conceivable that their unhindered ability to interact with C3/C3b might be crucial. Anti-band 3 NAb have an affinity for C3/C3b within their framework (31) and therefore can associate with CR1-bound C3b or, in the presence of native serum, may preferentially generate C3b₂-IgG complexes (30, 32), which then can interact bivalently with CR1. The involvement of CR1 as a receptor for pRBC on uninfected RBC is well established and of clinical relevance (36, 41), and monoclonal antibodies against the binding site for C3b on CR1 inhibit rosetting (42).

Although albumin was required for anti-band 3 NAb and factor D to fully induce de novo rosetting (Fig. 5), we propose that albumin acted as a shape-restoring protein but cannot exclude the possibility that it has a specific binding function. Anti-band 3 NAb and factor D were undoubtedly the primary mediators of de novo rosetting. Whether factor D cleaved a portion from a small fraction of a parasitic protein remains to be elucidated. A candidate protein would be PfEMP1, which carries several domains comprising potential binding sites for uninfected RBC (24). Constructs of the N-terminal DBL1 α domain of PfEMP1 bind to CR1 of uninfected RBC (41) and heparan sulfate (5, 51), and induced antibodies against DBL1 α disrupt rosettes (7).

ACKNOWLEDGMENTS

This work was supported by a competitive research grant to H.U.L. from the ETH (TH 0-20846-01), the Foundation of Swiss Life, and in

part to H.P.B. by the Emilia Guggenheim-Schnurr-Stiftung, Basel, Switzerland.

REFERENCES

- Barnum, S. R., M. A. Niemann, J. F. Kearney, and J. E. Volanakis. 1984. Quantitation of complement factor D in human serum by a solid-phase radioimmunoassay. *J. Immunol. Methods* **67**:303–309.
- Barragan, A., D. Spillmann, P. G. Kremsner, M. Wahlgren, and J. Carlson. 1999. Plasmodium falciparum: molecular background to strain-specific rosette disruption by glycosaminoglycans and sulfated glycoconjugates. *Exp. Parasitol.* **91**:133–143.
- Biggs, B. A., L. Gooze, K. Wycherley, W. Wollish, B. Southwell, J. H. Leech, and G. V. Brown. 1991. Antigenic variation in Plasmodium falciparum. *Proc. Natl. Acad. Sci. USA* **88**:9171–9174.
- Carlson, J., H. Helmbj, A. V. Hill, D. Brewster, B. M. Greenwood, and M. Wahlgren. 1990. Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies. *Lancet* **336**:1457–1460.
- Chen, Q., A. Barragan, V. Fernandez, A. Sundstrom, M. Schlichterle, A. Sahlen, J. Carlson, S. Datta, and M. Wahlgren. 1998. Identification of Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) as the rosetting ligand of the malaria parasite P. falciparum. *J. Exp. Med.* **187**:15–23.
- Chen, Q., A. Heddini, A. Barragan, V. Fernandez, S. F. Pearce, and M. Wahlgren. 2000. The semiconserved head structure of Plasmodium falciparum erythrocyte membrane protein 1 mediates binding to multiple independent host receptors. *J. Exp. Med.* **192**:1–10.
- Chen, Q., F. Pettersson, A. M. Vogt, B. Schmidt, S. Ahuja, P. Liljestrom, and M. Wahlgren. 2004. Immunization with PfEMP1-DBL1alpha generates antibodies that disrupt rosettes and protect against the sequestration of Plasmodium falciparum-infected erythrocytes. *Vaccine* **22**:2701–2712.
- Cheng, H. M., Y. F. Ngeow, and C. K. Sam. 1989. Heat inactivation of serum potentiates anti-cardiolipin antibody binding in ELISA. *J. Immunol. Methods* **124**:235–238.
- Clough, B., F. A. Atilola, J. Black, and G. Pasvol. 1998. Plasmodium falciparum: the importance of IgM in the rosetting of parasite-infected erythrocytes. *Exp. Parasitol.* **89**:129–132.
- Davis, A. E., III, C. Zalut, F. S. Rosen, and C. A. Alper. 1979. Human factor D of the alternative complement pathway. Physicochemical characteristics and N-terminal amino acid sequence. *Biochemistry* **18**:5082–5087.
- Fasler, S., F. Skvaril, and H. U. Lutz. 1988. Electrophoretic properties of human IgG and its subclasses on sodium dodecyl-sulfate-polyacrylamide gel electrophoresis and immunoblots. *Anal. Biochem.* **174**:593–600.
- Fearon, D. T., K. F. Austen, and S. Ruddy. 1974. Properdin factor D: characterization of its active site and isolation of the precursor form. *J. Exp. Med.* **139**:355–366.
- Fernandez, V., C. J. Treutiger, G. B. Nash, and M. Wahlgren. 1998. Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in Plasmodium falciparum malaria. *Infect. Immun.* **66**:2969–2975.
- Fernandez, V., and M. Wahlgren. 2002. Rosetting and autoagglutination in Plasmodium falciparum. *Chem. Immunol.* **80**:163–187.
- Flick, K., C. Scholander, Q. Chen, V. Fernandez, B. Pouvelle, J. Gysin, and M. Wahlgren. 2001. Role of nonimmune IgG bound to PfEMP1 in placental malaria. *Science* **293**:2098–2100.
- Fraser-Hurt, N., I. Felger, D. Edoh, S. Steiger, M. Mashaka, H. Masanja, T. Smith, F. Mbena, and H. P. Beck. 1999. Effect of insecticide-treated bed nets on haemoglobin values, prevalence and multiplicity of infection with Plasmodium falciparum in a randomized controlled trial in Tanzania. *Trans. R. Soc. Trop. Med. Hyg.* **93**(Suppl. 1):47–51.
- Giribaldi, G., D. Ulliers, F. Mannu, P. Arese, and F. Turrini. 2001. Growth of Plasmodium falciparum induces stage-dependent haemichrome formation, oxidative aggregation of band 3, membrane deposition of complement and antibodies, and phagocytosis of parasitized erythrocytes. *Br. J. Haematol.* **113**:492–499.
- Hasselaar, P., D. A. Triplett, A. LaRue, R. H. Derksen, L. Blokzijl, P. G. de Groot, D. R. Wagenknecht, and J. A. McIntyre. 1990. Heat treatment of serum and plasma induces false positive results in the antiphospholipid antibody ELISA. *J. Rheumatol.* **17**:186–191.
- Hidayat, A. A., R. M. Nalbandian, D. W. Sammons, J. A. Fleischman, and T. E. Johnson. 1993. The diagnostic histopathologic features of ocular malaria. *Ophthalmology* **100**:1183–1186.
- Ho, M., T. M. Davis, K. Silamut, D. Bunnag, and N. J. White. 1991. Rosette formation of Plasmodium falciparum-infected erythrocytes from patients with acute malaria. *Infect. Immun.* **59**:2135–2139.
- Hogh, B., E. Petersen, I. Crandall, A. Gottschau, and I. W. Sherman. 1994. Immune responses to band 3 neoantigens on Plasmodium falciparum-infected erythrocytes in subjects living in an area of intense malaria transmission are associated with low parasite density and high hematocrit value. *Infect. Immun.* **62**:4362–4366.
- Jeleszarova, E., A. Luginbühl, and H. U. Lutz. 2003. C3b2-IgG complexes retain dimeric C3 fragments at all levels of inactivation. *J. Biol. Chem.* **278**:51806–51812.
- Jeleszarova, E., and H. U. Lutz. 2005. IgG naturally occurring antibodies stabilize and promote the generation of the alternative complement pathway C3 convertase. *Mol. Immunol.* **42**:1393–1403.
- Kyes, S., P. Horrocks, and C. Newbold. 2001. Antigenic variation at the infected red cell surface in malaria. *Annu. Rev. Microbiol.* **55**:673–707.
- Lesavre, P. H., T. E. Hugli, A. F. Esser, and H. J. Muller-Eberhard. 1979. The alternative pathway C3/C5 convertase: chemical basis of factor B activation. *J. Immunol.* **123**:529–534.
- Lukacovic, M. F., M. B. Feinstein, R. I. Sha'afi, and S. Perrie. 1981. Purification of stabilized band 3 protein of the human erythrocyte membrane and its reconstitution into liposomes. *Biochemistry* **20**:3145–3151.
- Lutz, H. U., F. Bussolino, R. Flepp, S. Fasler, P. Stammer, M. D. Kazatchkine, and P. Arese. 1987. Naturally occurring anti-band-3 antibodies and complement together mediate phagocytosis of oxidatively stressed human erythrocytes. *Proc. Natl. Acad. Sci. USA* **84**:7368–7372.
- Lutz, H. U., S. Fasler, P. Stammer, F. Bussolino, and P. Arese. 1988. Naturally occurring anti-band 3 antibodies and complement in phagocytosis of oxidatively-stressed and in clearance of senescent red cells. *Blood Cells* **14**:175–203.
- Lutz, H. U., R. Flepp, and G. Stringaro-Wipf. 1984. Naturally occurring autoantibodies to exoplasmic and cryptic regions of band 3 protein, the major integral membrane protein of human red blood cells. *J. Immunol.* **133**:2610–2618.
- Lutz, H. U., and E. Jeleszarova. 2006. Complement amplification revisited. *Mol. Immunol.* **43**:2–12.
- Lutz, H. U., M. Nater, and P. Stammer. 1993. Naturally occurring anti-band 3 antibodies have a unique affinity for C3. *Immunology* **80**:191–196.
- Lutz, H. U., P. Stammer, and S. Fasler. 1993. Preferential formation of C3b-IgG complexes in vitro and in vivo from nascent C3b and naturally occurring anti-band 3 antibodies. *J. Biol. Chem.* **268**:17418–17426.
- Lutz, H. U., P. Stammer, and E. A. Fischer. 1990. Covalent binding of detergent-solubilized membrane glycoproteins to 'Chemobond' plates for ELISA. *J. Immunol. Methods* **129**:211–220.
- Lutz, H. U., and G. Wipf. 1982. Naturally occurring autoantibodies to skeletal proteins from human red blood cells. *J. Immunol.* **128**:1695–1699.
- Mihaescu, S., R. Lenkei, and V. Ghetie. 1981. Radioimmunoassay of anti-albumin autoantibodies in human sera. *J. Immunol. Methods* **42**:187–193.
- Moulds, J. M., L. Kassambara, J. J. Middleton, M. Baby, I. Sagara, A. Guindo, S. Coulibaly, D. Yalcouye, D. A. Diallo, L. Miller, and O. Doumbo. 2000. Identification of complement receptor one (CR1) polymorphisms in west Africa. *Genes Immun.* **1**:325–329.
- Paul, F., S. Roath, D. Melville, D. C. Warhurst, and J. O. Osianaya. 1981. Separation of malaria-infected erythrocytes from whole blood: use of a selective high-gradient magnetic separation technique. *Lancet* **2**:70–71.
- Reichstein, E., and R. Blostein. 1975. Arrangement of human erythrocyte membrane proteins. *J. Biol. Chem.* **250**:6256–6263.
- Reliene, R., M. Mariani, A. Zanella, W. H. Reinhart, M. L. Ribeiro, E. M. del Giudice, S. Perrotta, A. Iolascon, S. Eber, and H. U. Lutz. 2002. Splenectomy prolongs in vivo survival of erythrocytes differently in spectrin/ankyrin- and band 3-deficient hereditary spherocytosis. *Blood* **100**:2208–2215.
- Riganti, M., E. Pongponratn, T. Tegoshi, S. Looareesuwan, B. Punpoowong, and M. Aikawa. 1990. Human cerebral malaria in Thailand: a clinico-pathological correlation. *Immunol. Lett.* **25**:199–205.
- Rowe, J. A., J. M. Moulds, C. I. Newbold, and L. H. Miller. 1997. P. falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature* **388**:292–295.
- Rowe, J. A., S. J. Rogerson, A. Raza, J. M. Moulds, M. D. Kazatchkine, K. Marsh, C. I. Newbold, J. P. Atkinson, and L. H. Miller. 2000. Mapping of the region of complement receptor (CR) 1 required for Plasmodium falciparum rosetting and demonstration of the importance of CR1 in rosetting in field isolates. *J. Immunol.* **165**:6341–6346.
- Scholander, C., C. J. Treutiger, K. Hultenby, and M. Wahlgren. 1996. Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. *Nat. Med.* **2**:204–208.
- Sherman, I. W., S. Eda, and E. Winograd. 2003. Cytoadherence and sequestration in Plasmodium falciparum: defining the ties that bind. *Microbes Infect.* **5**:897–909.
- Sommer, E. A., J. Black, and G. Pasvol. 2000. Multiple human serum components act as bridging molecules in rosette formation by Plasmodium falciparum-infected erythrocytes. *Blood* **95**:674–682.
- Trager, W., and J. B. Jensen. 1976. Human malaria parasites in continuous culture. *Science* **193**:673–675.
- Treutiger, C. J., I. Hedlund, H. Helmbj, J. Carlson, A. Jepson, P. Twumasi, D. Kwiatkowski, B. M. Greenwood, and M. Wahlgren. 1992. Rosette formation in Plasmodium falciparum isolates and anti-rosette activity of sera from Gambians with cerebral or uncomplicated malaria. *Am. J. Trop. Med. Hyg.* **46**:503–510.
- Treutiger, C. J., C. Scholander, J. Carlson, K. P. McAdam, J. G. Raynes, L. Falksveden, and M. Wahlgren. 1999. Rouleaux-forming serum proteins are involved in the rosetting of Plasmodium falciparum-infected erythrocytes. *Exp. Parasitol.* **93**:215–224.
- Udomsangpetch, R., B. Wahlin, J. Carlson, K. Berzins, M. Torii, M. Aikawa, P. Perlmann, and M. Wahlgren. 1989. Plasmodium falciparum-infected erythrocytes form spontaneous erythrocyte rosettes. *J. Exp. Med.* **169**:1835–1840.

50. Uhlemann, A. C., T. Staalsoe, M. Q. Klinkert, and L. Hviid. 2000. Analysis of *Plasmodium falciparum*-infected red blood cells. *MACS & More* **4**:7–8. <http://www.miltenyibiotec.com>.
51. Vogt, A. M., A. Barragan, Q. Chen, F. Kironde, D. Spillmann, and M. Wahlgren. 2003. Heparan sulfate on endothelial cells mediates the binding of *Plasmodium falciparum*-infected erythrocytes via the DBL1alpha domain of PfEMP1. *Blood* **101**:2405–2411.
52. Winograd, E., and I. W. Sherman. 1989. Naturally occurring anti-band 3 autoantibodies recognize a high molecular weight protein on the surface of *Plasmodium falciparum* infected erythrocytes. *Biochem. Biophys. Res. Commun.* **160**:1357–1363.
53. Yoshida, H., H. Ishikawa, S. Honda, Y. Miura, and M. Ogata. 1992. False positive reaction of heat-inactivated sera in enzyme-linked immunosorbent assay for antibody to hepatitis C virus. *Fukushima J. Med. Sci.* **38**:35–41.

Editor: W. A. Petri, Jr.