Plasmodium falciparum Erythrocyte Rosetting Is Mediated by Promiscuous Lectin-like Interactions

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Summary

Herein we describe an assay that was developed to quantitate the binding of normal red blood cells (RBC), labeled with carboxy fluorescein diacetate (C-FDA), to rosetting *Plasmodium falciparum*–infected RBC. The binding of RBC obtained from various animal species or humans to different strains or clones of rosetting *P. falciparum*–infected RBC was studied. A strain-specific preference of rosetting was observed for either blood group A/AB or B/AB RBC for all parasites tested. The higher affinity of rosette binding of blood group A, B, or AB vs. O RBC was reflected in larger rosettes when a given parasite was grown in RBC of the preferred blood group. The small size of the rosettes formed when *P. falciparum* was grown in blood group O RBC may be the in vitro correlate of the relative protection against cerebral malaria afforded by belonging to blood group O rather than to blood group A or B. Rosettes of a blood group A–preferring parasite could be completely disrupted by heparin only when grown in blood group O or B RBC, but not when grown in blood group A RBC. Similarly, the rosettes of a blood group B–preferring parasite could be more easily disrupted by heparin when grown in blood group O or A RBC than when grown in blood group B RBC. Several different saccharides inhibited rosetting of group O RBC, including two monosaccharides that are basic components of heparin. The rosetting of the same parasites grown in blood group A or B RBC was less sensitive to heparin and was specifically inhibited only by the terminal mono- and trisaccharides of the A and the B blood group antigens, the H disaccharide, and fucose. Our results suggest that rosetting is mediated by multiple lectin-like interactions, the usage of which rely on the parasite phenotype and whether the receptors are present on the host cell or not.

Erythrocyte rosetting (1–4), the spontaneous binding of uninfected to *Plasmodium falciparum*–infected RBC, has previously been hypothesized to play a role in the pathogenesis of cerebral malaria (1, 5–7). "Rosettins," strain-specific low molecular weight polypeptides derived from the parasite and located on the surface of the infected erythrocyte, are believed to mediate rosetting (8, 8a), however, very little is known about the receptors on the uninfected erythrocyte. We therefore set out to investigate the erythrocyte requirements of rosetting and the involved receptors.

Materials and Methods

P. falciparum Strains/Isolates. Five different *P. falciparum* strains/isolates were cultured according to standard procedures with 10% AB^+ Rh^+ serum added to the buffered medium. The R^+PA1 strain is a cloned rosetting parasite obtained from the Palo Alto strain (Uganda) (3). The isolates TM178, TM180, and TM284 were isolated in 1990 from Thai patients with acute malaria and were provided by Dr. Sodsri Thaithong (Chulalongkorn University, Bangkok, Thailand). The rosetting Malayan Camp (MCR^+) parasites (9) were provided by Dr. R. J. Howard (Affymax Research Institute, Palo Alto, CA).

Red Blood Cells. Human blood was drawn into heparinized tubes from healthy Swedish blood donors not exposed to *P. falciparum* malaria. ABO blood group typing was carried out using a hemagglutination technique with mAbs (BioCarb, Lund, Sweden). Animal blood was obtained from healthy BALB/C mice, Sprague Dawley rats, New Zealand White rabbits, Siberian hamsters, and guinea pigs from our own animal facility; from Swedish land race breed sheep, Swedish land race breed goats, Yorkshire pigs, Swedish Fresian cattle, and Swedish trotters from The National Veterinary Institute, Uppsala, Sweden; and from *Aotus trivirgatus* and *Saimiri sciureus* monkeys from the Institute Pasteur (Cayenne, French Guiana).

Mono- and Oligosaccharides. The blood group O (H antigen) disaccharide [D-gal(1-3)α-L-fuc] and the blood group A trisaccharide [α-D-galNAc(1-3)β-D-gal(1-3)α-D-fuc], and the B trisaccharide [α-D-gal(1-3)β-D-gal(1-3)α-L-fuc], consisting of the terminal sugars of the H, A, and B blood group antigens, respectively, purified from urine and estimated to be homogeneous to ~95% as esti-

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mated by HPLC (10), were purchased from BioCarb Chemicals (Lund, Sweden) and from Accurate Chemical & Scientific Corp. (Westbury, NY). The monosaccharides D-glucose (glc), D-glucosamine (glcN), *N*-acetyl-D-glucosamine (glcNAc), D-galactose (gal), D-galactosamine (galN), *N*-acetyl-D-galactosamine (galNAc), D-mannose (man), D-mannosamine (manN), *N*-acetyl-D-mannosamine (manNAc), and D-fucose (fuc) were all purchased from Sigma Chemical Co. (St. Louis, MO) and were 90–95% pure.

Labeling of RBC with Carboxy-Fluorescein Diacetate (C-FDA).[1] RBC were labeled with C-FDA, a membrane-permeable nonfluorescent fatty acid ester that, after entering the cell, is hydrolyzed to produce free polar carboxy-fluorescein, a fluorescent compound (11). A stock solution of C-FDA in acetone (15 mg/ml) was diluted 50 times in malaria culture medium (MCM) to a final concentration of 300 μg/ml. After washing RBC three times in Tris-Hanks solution, the centrifugated cell pellet (50 μl) was suspended in 1 ml of the C-FDA labeling solution. After labeling for 30–45 min at 37°C, the RBC were washed twice in Tris-Hanks solution and resuspended in serum containing MCM.

Assessment of Rosette Formation and Competition with C-FDA-labeled RBC. An assay was developed where C-FDA-labeled RBC were allowed to compete with unlabeled RBC in order to measure their relative capacity to form rosettes. Rosettes were, as previously, defined as *P. falciparum*–infected RBC that had bound two or more noninfected RBC (8).

Existing rosettes in a *P. falciparum* culture, grown at a 5% hematocrit in blood group O RBC, were disrupted by addition of heparin (Kabi Pharmacia AB, Stockholm, Sweden); the concentration depending on the strain tested (50 IU/ml for R^+PA1 and 1,000–1,500 IU/ml for TM178, TM180, TM284, and MCR^+), as the sensitivity of rosettes to heparin varies (12). The cultures were checked by UV microscopy to ensure that disruption of all rosettes was complete. 50-μl aliquots of the unlabeled heparin-treated culture were mixed with 5% C-FDA-labeled RBC in heparin. While the concentration of the C-FDA-labeled RBC added in the initial validation experiments varied, an equal volume of labeled and unlabeled RBC was always used in the subsequent standardized experiments. The mixture was washed twice in RPMI, to remove heparin, and subsequently resuspended in serum containing MCM. After incubation for 30 min, the cell suspension was mixed with a small amount of acridine orange (for identification of the parasitized cells), mounted on glass slides, and studied in incident UV light (40× lens and 10× ocular; Leitz Laborlux K microscope, Wetzlar, Germany). The proportion of labeled RBC in the rosettes was calculated and compared with the proportion of labeled cells in the whole erythrocyte population. The difference in the percentage labeled cells in the rosettes and in the total labeled/unlabeled erythrocyte mixture was expressed as a relative value of increase/decrease in the rosette-forming capacity of the labeled red cells.

In a slightly modified assay, a 50-μl aliquot of a rosetting culture grown in blood group O RBC was mixed with C-FDA-labeled uninfected RBC (blood group O, A, or B) in serum containing MCM. The rosettes were then disrupted mechanically by drawing the RBC mixture through a narrow-gauge injection needle six to eight times (Ø = 0.65 mm; Termo, Leuven, Belgium), a procedure known to cause adequate disruption of the rosettes followed by prompt reformation. Mixing with acridine orange and assessment of rosette formation was subsequently made after 30 min, as described above.

[1] *Abbreviations used in this paper:* C-FDA, carboxy-fluorescein diacetate; MCM, malaria culture medium.

Inhibition of Rosette Reformation by Mono- or Oligosaccharides. Mono- and oligosaccharides were added in various concentration to 50-μl aliquots of a rosetting strain (R^+PA1 or TM284) cultured in blood group O, A, or B RBC. Disruption of rosettes was subsequently made mechanically as described above, and after spontaneous reformation of the rosettes, the rosetting rate of each sample was calculated as before (8). Inhibition was estimated by comparing the rosetting rate of each sample with that of a sample without additives.

Rosette Formation of Parasites Grown in RBC of Different ABO Blood Groups. The strains R^+PA1 and TM284 grown in O^+ RBC were subcultivated in group A, B, AB, or O RBC that had been drawn the same day and stored for 4–5 d at +4°C before use. The cultures were subcultivated another two times the following week by adding 80–90% fresh RBC, thereby diluting away the original O^+ RBC and obtaining comparable parasitaemias (3–6%). Assays were performed 48–72 h after the third subcultivation. The rosetting rate, the number of infected RBC per rosette, and the number of uninfected RBC bound to each infected erythrocyte were calculated by counting 2 × 50 fields of vision.

Disruption of Rosettes with Heparin. Heparin was added in various concentrations (0.025–2,000 IU/ml) to 50-μl aliquots of the strains R^+PA1 and TM284 cultivated in group A, B, or O blood and incubated in a 96-well microtiter plate for 30 min before assessment of rosetting. The rosetting rate of each well was compared with that of a control with MCM as additive.

Results

Both C-FDA Assays Are Suitable for Testing of the Erythrocyte Rosette Binding Capacity. A validation test of the C-FDA assays was performed where C-FDA-labeled RBC were competing in rosette formation with unlabeled RBC from the same O Rh^+ donor (Fig. 1, *A* and *B*). The gross erythrocyte morphology was not altered after labeling or during the assay. There was a good correlation between the proportion of labeled/unlabeled cells in the mixed RBC population in the culture and the proportion in the rosettes (Fig. 1 *B*).

The Rosette-forming Capacity Varies with the Animal Species from which the RBC Are Obtained. The rosette binding capacity of RBC obtained from various animal species was tested using the R^+PA1 and TM284 strains grown in human O Rh^+ RBC. Hamster and rat RBC bound to a similar degree as human O RBC, while mouse, rabbit, and monkey (*Aotus* and *Saimiri*) RBC were slightly decreased in their binding capacity. No RBC from the other animal species tested did form rosettes with parasitized human RBC (not shown).

The Rosette Binding Capacity of RBC of Different ABO Blood Groups Varies. The rosetting ability of RBC from 52 donors carrying different ABO blood group antigens was tested with O Rh^+ RBC parasitized with the R^+PA1 strain. A distinct pattern of binding preference appeared (Fig. 2 *A*) where RBC from A and AB donors were preferred over those of the O donors (mean, +39% and +36%, respectively). The group O RBC tested gave the same binding as the control O Rh^+ RBC (mean ± 0), while group B RBC showed a slight mean increase of 4%. Four donors belonging to blood group A, subtype A_1 were also compared with four donors belonging to subtype A_2, and a stronger mean increase in rosette-forming ability was found in the former group (mean, +46%

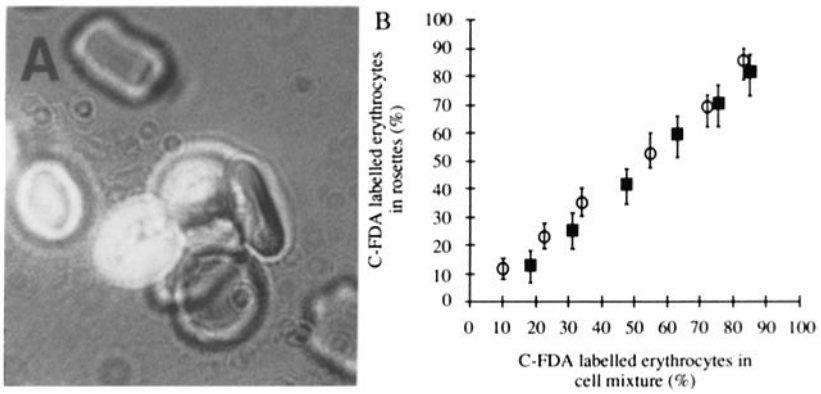

Figure 1. (*A*) C-FDA-labeled and unlabeled RBC bound in a rosette to a *P. falciparum*–infected erythrocyte (see Materials and Methods); (*B*) comparison of the percentage of C-FDA-labeled RBC in the mixed labeled/unlabeled cell culture and the percentage of C-FDA-labeled RBC in R^+PA1 rosettes after disrupting and reforming rosettes. (○) Disruption with heparin; (■) mechanical disruption in a narrow-gauge needle. Both infected and uninfected, C-FDA-labeled or unlabeled RBC were from the same donor belonging to blood group O Rh^+. Values given are means ± SD.

vs. +28%), however, with great individual differences (not shown). RBC from an individual of the Bombay phenotype (O_h), with deficient stem chain, thus lacking the terminal fucose present on the O_H RBC, was also tested and exhibited an ability to form rosettes similar to that of group O blood (99% of a group O^+ control donor). No preference for Rh phenotype was seen (Fig. 2 *A*).

The ABO Blood Group Preference Varies with the P. falciparum Strain. To investigate possible strain differences in the blood group preference of rosetting, four additional strains/isolates were tested and compared with the R^+PA1 strain. All four strains/isolates were cultured in O^+ RBC and tested with RBC drawn from the same 13 donors (MCR^+ was tested with 12 donors) carrying different ABO blood group antigens. The R^+PA1, as already known, and two other strains, TM178 and MCR^+, exhibited an A/AB preference in rosette formation while there was a B/AB preference of the strains TM180 and TM284 (Fig. 2 *B*).

The Number of Adhering RBC in Rosettes Depends on the ABO Blood Group. When the R^+PA1 strain, with a group A/AB rosetting preference, was cultured in RBC of blood group A or AB, the average rosette formed in the culture was larger than when the same parasite strain was propagated in group B or O RBC. This was due to a larger number of uninfected RBC bound to each parasite-infected erythrocyte (Table 1). A small increase of rosette size was also seen when grown in group B RBC compared with O RBC. A similar pattern was observed for the blood group B/AB–preferring TM284 strain such that the rosettes were larger in group B or AB RBC as compared with A or O RBC. No significant difference was seen in rosetting rate (not shown) nor the number of parasite-infected RBC per rosette.

Mono- and Oligosaccharides Inhibit Reformation of Mechanically Disrupted Rosettes. Mono- and oligosaccharides were added to cultures of R^+PA1 (grown in either O or A RBC) or TM284 (grown in either O or B RBC) followed by mechanical disruption of rosettes. The A- and B-trisaccharides (α-D-galNAc[1-3]β-D-gal[1-3]α-D-fuc and α-D-gal [1-3]β-D-gal[1-3]α-L-fuc, respectively) dose dependently inhibited the reformation of rosettes of R^+PA1 grown in A RBC and TM284 grown in B RBC, respectively (Fig. 3, *B* and *D*). Although there was an effect with these saccharides on rosette reformation when the parasites were grown in group O RBC, it was only pronounced with the TM284 strain (Fig. 3, *A* and *C*). The A-monosaccharide (galNAc) also gave some inhibition of R^+PA1 rosettes in group A RBC, while no significant effect on the rosetting rate of TM284 rosettes in group B blood was seen with the B-monosaccharide (gal). Some inhibitory effect was also seen with fuc and the H-disaccharide (D-gal[1-3]α-L-fuc) of rosettes grown in all blood groups. The monosaccharides glcN and manN, and to some extent galN and glcNAc, gave inhibition of group O RBC but had little or no effect on reformation of rosettes of group A or B RBC. The results of the blood group saccharide inhibition studies above were confirmed using a competition assay where infected RBC of a blood group A– or B–preferring parasite, grown in group O RBC, were mixed with uninfected C-FDA-labeled group A or B RBC and preincubated with the different saccharides (not shown).

The Sensitivity of Rosettes to Heparin Depends on the ABO Blood Group of the RBC in which the Parasite Is Propagated. Disruption with heparin of preformed rosettes in culture was studied with the blood group A–preferring strain R^+PA1 and the blood group B–preferring strain TM284 cultivated in group O, A, or B blood. When R^+PA1 parasites were cultivated in group O RBC, the heparin concentrations needed to disrupt rosettes were as low as previously reported (8). Higher heparin concentrations were, however, needed to achieve disruption of R^+PA1 rosettes grown in blood group B RBC and in particular in group A RBC (Fig. 4 *A*). When grown in RBC of the latter group, total disruption of rosettes could not be obtained using the ordinary disruption assay. However, when high concentrations of heparin (1,000–1,500 IU/ml) were used and after washing of the rosettes twice in serum-free medium (RPMI), the rosettes did not reappear (not shown). Similarly, higher concentrations of heparin were needed to disrupt TM284 rosettes when the parasite was grown in blood group B RBC as compared with group A, and in particular group O RBC (Fig. 4 *B*).

Discussion

The requirements for participation in rosette formation with human *P. falciparum*–infected RBC are fulfilled only by

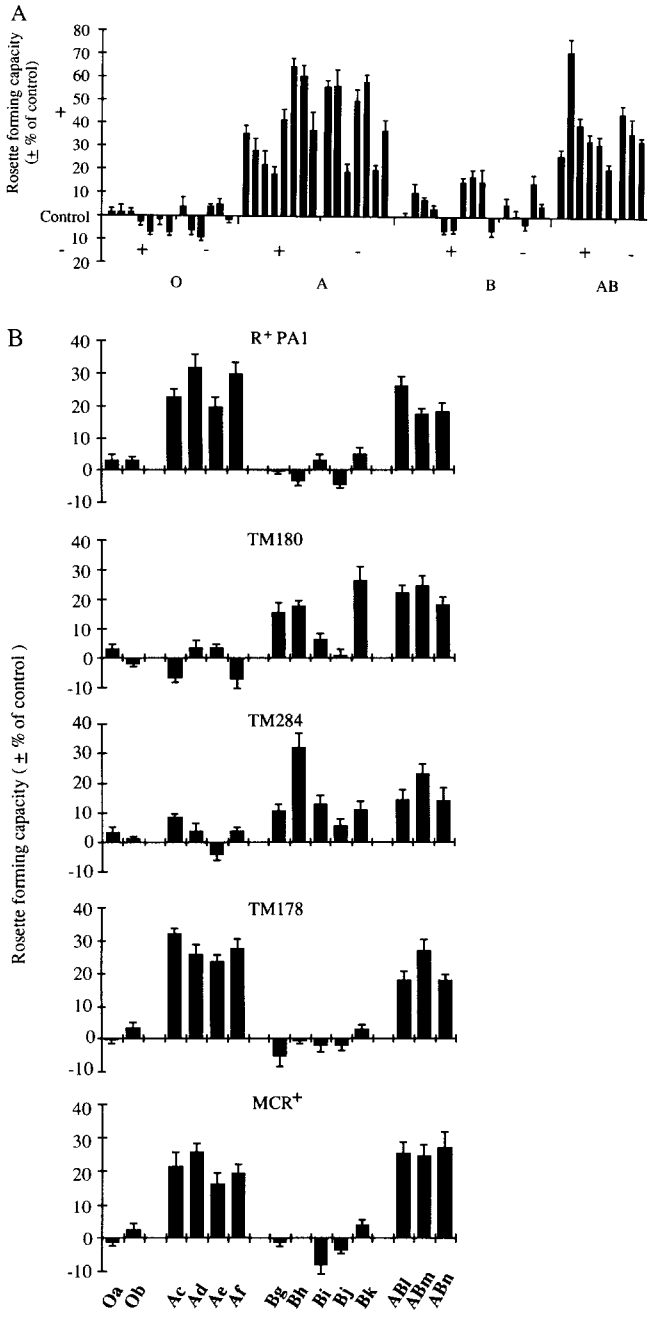

Figure 2. Rosetting capacity of RBC from donors belonging to different ABO/Rh blood groups. (*A*) The *P. falciparum* strain R+PA1 was cultivated in human blood group O Rh+ RBC. Shown is the increase or decrease in the rosette binding capacity of the C-FDA-labeled RBC from 52 donors compared with the RBC of the blood group O Rh+ control donor used as index (baseline). Values given are means ± SD. (*B*) The binding of blood group O Rh+ control RBC, parasitized with one of five different *P. falciparum* strains/isolates (R+PA1, TM180, TM284, TM178, MCR+), was used as baseline for the rosetting capacity of each parasite. The test RBC used with each strain were from the same 13 donors (*a–n*). RBC from donor B_h were not tested with the strain MCR+. Values given are means ± SD.

Table 1. *The Number of RBC in Rosettes Depending on the ABO Blood Group*

Strain	Blood group	Infected RBC/ rosette	Uninfected RBC/ infected RBC	
				mean
R+PA1	O Rh+	1.6	2.2	
	O Rh+	1.2	2.8	
	O Rh+	1.3	2.6	2.5
	A Rh+	1.2	4.1	
	A Rh+	1.1	5.3	
	A Rh+	1.4	4.4	4.6
	B Rh+	1.3	3.0	
	B Rh+	1.4	2.6	2.8
	AB Rh+	1.2	4.2	
	AB Rh+	1.2	4.6	4.4
TM284	O Rh+	1.1	3.3	
	O Rh+	1.1	3.2	3.3
	A Rh+	1.2	3.4	3.4
	B Rh+	1.1	4.1	
	B Rh+	1.2	3.9	4.0
	AB Rh+	1.2	4.3	
	AB Rh+	1.3	4.0	4.2

A blood group A (R+PA1)- or blood group B (TM284)-preferring *P. falciparum* strain was grown in RBC from donors belonging to different ABO blood groups. Values given are averages of observations made after 24–30 h during two or three parasite life cycles.

RBC of some animal species (mouse, hamster, rat, rabbit, monkey), and e.g., sheep RBC do not form rosettes with either the R+PA1 or the TM284 parasites. The rosetting capacity of human RBC of different blood groups was also studied, and while we found no difference between RBC obtained from donors of the Rh+ or the Rh− phenotypes, a distinct pattern of preference of blood groups A and AB vs. blood groups O and B was observed with the R+PA1 clone, and the TM178 and Malayan Camp strains. In contrast a blood group B/AB preference was seen with the TM284 and TM180 strains. In a separate extended study subsequently performed in the Gambia, an A or a B blood group preference was found with all the seven fresh clinical isolates analyzed (13). Thus, all wild and in vitro propagated parasites studied so far have exhibited a preference of binding to either A/AB or B/AB RBC as compared with blood group O.

Rosettes were recently reported to be highly resistant to shear stress and were not significantly disrupted by stress of the order seen in arterial flow (14). In addition, rosettes were shown to cause considerable resistance of flow into a capillary-

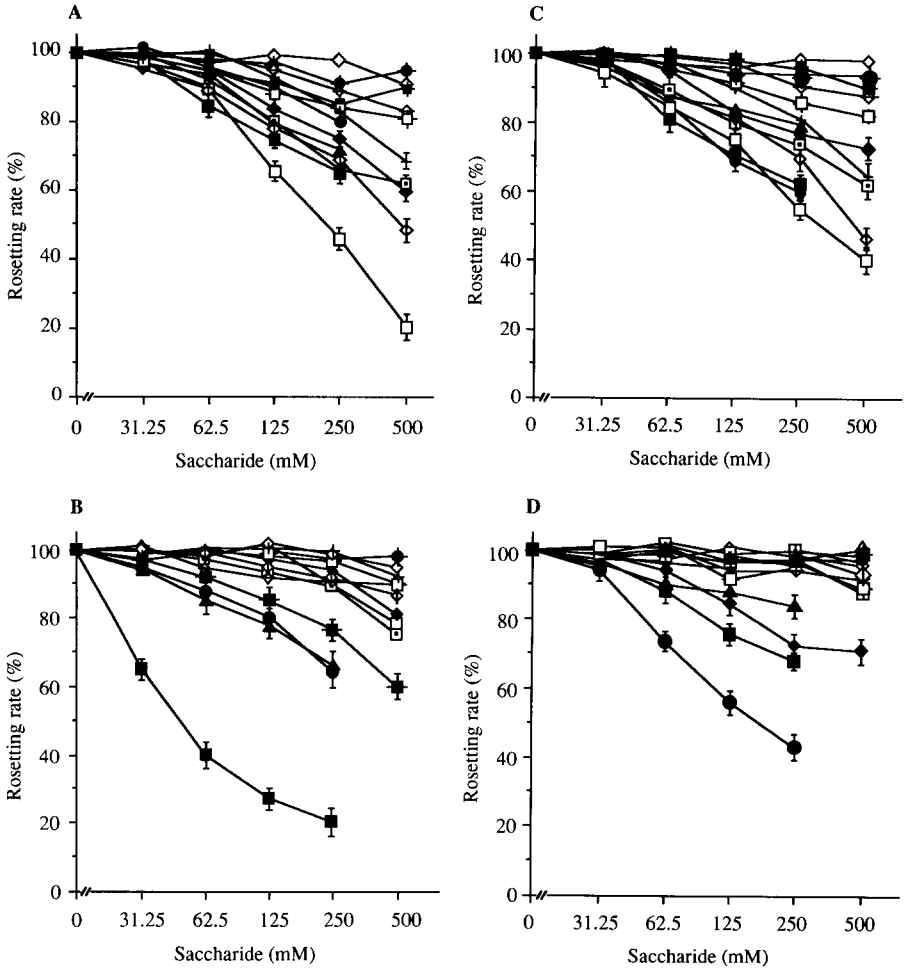

Figure 3. Inhibitory effect of various mono- and oligosaccharides on reformation of mechanically disrupted rosettes. The group A–preferring strain R+PA1 grown in (*A*) blood group O and (*B*) group A RBC. The group B–preferring strain TM284 grown in (*C*) blood group O and (*D*) group B RBC. (■) A trisaccharide; (●) B trisaccharide; (▲) H disaccharide; (◆) fuc; (▣) galNAc; (◈) gal; (⊡) glc; (□) glcN; (⧈) glcNAc; (+) galN; (◇) man; (◊) manN; (◈) manNAc. Values given are means ± SD. The SD values of weakly inhibitory or noninhibitory saccharides are not shown.

sized pipette. This resistance was dramatically augmented by increasing the size of the rosettes, predicting a significant impairment of flow into small vessels caused by large rosettes (14). This is particularly interesting since we have observed that the higher affinity of binding of A, B, or AB RBC to *P. falciparum*–infected RBC was reflected in a larger size of the rosettes when a parasite was grown in RBC carrying the blood group preferred by that strain (e.g., the rosettes of the A/AB-preferring parasite R+PA1 were almost twice the size when grown in blood group A or AB RBC as compared with blood group O RBC). Similarily, the rosettes of the blood group B–preferring parasite TM284 were larger when grown in blood group B or AB as compared with when grown in blood group O or A RBC. Malaria in individuals belonging to a blood group "preferred" by the infecting *P. falciparum* strain may thus lead to the formation of large rosettes and an increased tendency of microvascular blockage. This finding fits well with the recent observation that patients that carry the blood group O antigens are relatively protected against cerebral malaria as compared with patients carrying the A and B antigens (A. Hill, et al., manuscript submitted for publication). Why the blood group O gene, although common in malarious areas, has only attained a high fixation rate in some endemic areas may be explained by a switch in rosetting receptor preferences and/or the simultaneous importance of other, ABO-independent, mechanisms of protection against severe malaria. The influence on the ABO blood group distribution by mortality due to other diseases must also be acknowledged.

There was a striking difference in sensitivity of rosettes to both heparin and the saccharides when the two tested parasite strains were propagated in RBC carrying a "preferred" blood group as compared with when propagated in group O RBC or RBC of another nonfavored blood group (e.g., rosettes of the A/AB-preferring parasites R+PA1 were only disrupted to completion by heparin when grown in blood group O or B RBC but not when grown in blood group A RBC). Similarly, the rosettes of the blood group B–preferring parasite could more easily be disrupted by heparin when grown in A or O RBC than when grown in blood group B RBC. Moreover, rosettes in group O blood, highly sensitive to heparin, were inhibited by glcN and glcNAc, two basic constituents of heparin, while heparin-resistant rosetting was affected by neither of these saccharides. However, blood group O rosettes could also be inhibited to some extent by manN and galN, an effect seen neither with blood

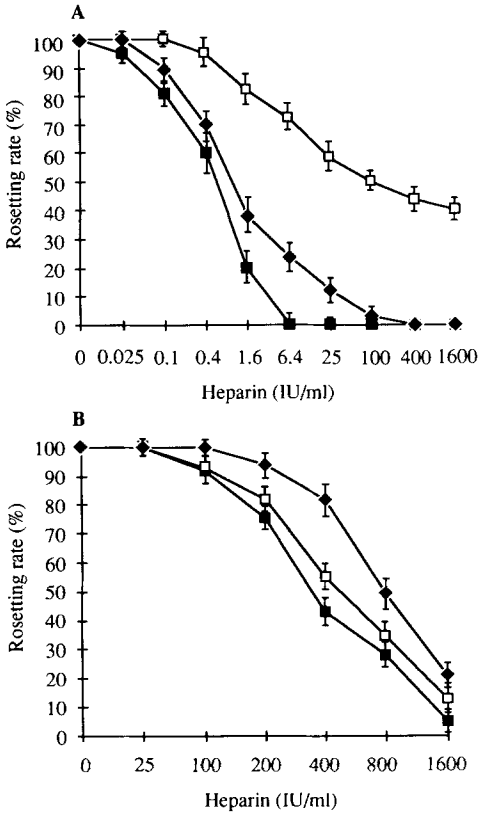

Figure 4. Disruption of erythrocyte rosettes with various concentrations of standard heparin when growing the *P. falciparum* parasites in RBC of different ABO blood groups. The effect of heparin on the rosetting rate of (*A*) the blood group A–preferring clone R$^+$PA1 cultured in A (□), B (◆), and O (■) RBC, and (*B*) the blood group B–preferring strain TM284 cultured in A (□), B (◆), and O (■) RBC are shown. Values given are means ± SD.

group A nor with blood group B rosettes. Taken together, it may be that heparin, when disrupting rosettes, interferes with interactions involving glcN and glcNAc, but other carbohydrate binding structures also seem to exist. A similar sensitivity of rosettes to glcN has also been observed previously (15). van Schravendijk et al. (16) recently found low amounts of CD36 on normal erythrocytes and showed its involvement as a receptor for rosetting of the MCR$^+$ strain (9). However, this strain is also a blood group A–preferring parasite, a fact that makes us wonder if it is not the sugars on CD36, rather than the peptide backbone, that constitute the rosetting receptor.

While the rosettes formed by blood group O RBC may be inhibited by several different saccharides, the binding of A or B RBC to *P. falciparum*–infected RBC could strain specifically and only be blocked with constituents of the terminal trisaccharides of the A or B blood group antigens. These data thus suggest that the A and B blood group antigens present on band 3, band 4.5, and on glycolipids (17) constitute specific receptors for rosette formation. Taken together, the erythrocyte adhesion in rosette formation seems to be mediated by diverse lectin-like interactions of the rosettins to several carbohydrate moieties, some that are constituents of heparin, and others that are ABO blood group antigens.

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