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Sulfated Polyanions Inhibit Invasion of Erythrocytes by Plasmodial Merozoites and Cytoadherence of Endothelial Cells to Parasitized Erythrocytes

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Sulfated proteoglycans have been shown to be involved in the binding of sporozoites of malaria parasites to hepatocytes. In this study, we have evaluated the effect of sulfated glycosaminoglycans on the invasion of erythrocytes by *Plasmodium falciparum* merozoites and cytoadherence of parasitized erythrocytes (PRBC) to endothelial cells. Invasion of erythrocytes by HB3EC-6 (an HB3 line selected for high binding to endothelial cells) was inhibited by dextran sulfate 500K, dextran sulfate 5K, sulfatides, fucoidan, and heparin but not by chondroitin sulfate A. With the exception of sulfatides, the invasion-inhibitory effect was not mediated by killing of parasites. Cytoadherence of HB3EC-6 to human microvascular endothelial cells (HMEC-1) and HB3C32-6 (an HB3 line selected for high binding to C32 melanoma cells) to C32 melanoma cells was also inhibited by these sulfated glycoconjugates. The highly sulfated dextran sulfate 500K had the highest inhibitory effect on both invasion and cytoadherence. Both unsulfated dextran 500K and hyaluronic acid had no significant effect on invasion or cytoadherence, whereas the positively charged protamine sulfate promoted cytoadherence. Because preincubation of PRBC with sulfated glycosaminoglycans and treatment of target cells with heparinase had no significant inhibition on cytoadherence, it is unlikely that sulfated glycoconjugates are used directly by endothelial cells as cytoadhesion receptors. In an in vivo experiment, we found that the administration of dextran sulfate 500K to CBA/Ca mice infected with *Plasmodium berghei* ANKA reduced parasitemia and delayed the death associated with anemia. These observations suggest that sulfated polyanions inhibit the invasion of erythrocytes by merozoites and cytoadherence of PRBC to endothelial cells by increasing the negative repulsive charge and sterically interfering with the ligand-receptor interaction after binding to target cells.

Plasmodium falciparum is the most pathogenic human malaria parasite and responsible for the majority of malaria-related morbidity and mortality. It has been suggested that the high pathogenicity of this parasite is due to the invasion of mature erythrocytes by the parasite and the adherence (sequestration) of erythrocytes infected with late trophozoites and schizonts (parasitized erythrocytes [PRBC]) to the endothelium of capillaries and postcapillary venules (18). Other human malaria parasites, such as *Plasmodium vivax*, only invade a small population of erythrocytes (reticulocytes) and do not sequester, thus rarely causing fatal diseases.

Despite the enormous research effort in this area, the mechanisms of both merozoite invasion and PRBC sequestration are not fully understood. Several proteins, such as merozoite surface protein 1 (MSP-1) and the 175-kDa erythrocyte-binding antigen (EBA-175) on merozoites and glycophorins A and B on erythrocytes, have been described to be involved in the invasion of erythrocytes by *P. falciparum* (10, 31). Likewise, several adhesion molecules, such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1), sequestrin and modified band 3 on PRBC, intercellular cytoadhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), CD36, and thrombospondin and E-selectin on endothelial cells, are involved in the sequestration of PRBC (24). The detailed process of interactions between ligands and receptors, however, is not clear. Additional ligands and receptors have also been suggested to be involved in both invasion and sequestration (6, 10).

In this study, we have tested the effect of sulfated glycoconjugates (positively or negatively charged) on the invasion of erythrocytes by *P. falciparum* and adherence of PRBC to endothelial cells. Some of these negatively charged polysaccharides, glycosaminoglycans, and glycolipids (such as heparan sulfate and chondroitin sulfate) are exposed in large quantities on the surface of endothelial cells and hepatocytes as the side chains of proteoglycans (4, 12), thus contributing to the net negative surface charge of these cells. Because sulfated glycoconjugates are used by many infectious agents, including plasmodial sporozoites, as cytoadhesion receptors, we have also assessed the possibility that these sulfated glycoconjugates are receptors for the adhesion of PRBC to endothelial cells.

MATERIALS AND METHODS

Reagents and cell lines. All glycoconjugates (dextran 500,000 [500K], dextran sulfate 500K, dextran sulfate 5K, heparin [176 USP units/mg], sulfatides, keratan sulfate, fucoidan, chondroitin sulfate A, heparan sulfate, and hyaluronic acid) were obtained from Sigma (St. Louis, Mo.), as were heparinase and the arginine-rich protein protamine sulfate. [^{3}H]hypoxanthine was from Amersham Life Science (Buckinghamshire, England). Gelatin (275 bloom) was purchased from Fisher Scientific (Pittsburgh, Pa.). Human platelet CD36 was a gift of Narendra N. Tandon.

Two cell lines, HMEC-1 and C32 melanoma cells, were used in the in vitro cytoadhesion assay. HMEC-1 is an endothelial cell line isolated from human dermal microvascular endothelium and immortalized by transfection with simian

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virus 40 large T antigen (1). Human C32 amelanotic melanoma cells were obtained from the American Type Culture Collection (Rockville, Md.).

Parasite culture. *P. falciparum* parasites were cultured in O^+ human erythrocytes (33). Parasitemia was maintained up to 5% and hematocrit to 10%. Late trophozoite- and early schizont-infected erythrocytes (PRBC) were concentrated by flotation in 0.5% gelatin for 1 h (15) before they were used for invasion inhibition assays and cytoadhesion assays.

Two HB3 descendant lines (HB3EC-6 and HB3C32-6) of *P. falciparum* were used for invasion and cytoadhesion studies. HB3EC-6 and HB3C32-6 were selected by panning the parental HB3 parasite strain six times over HMEC-1 and C32 melanoma cells, respectively. These two HB3 lines bind intensively to HMEC-1, C32 melanoma cells, and immobilized CD36.

***P. falciparum* growth and invasion inhibition assays.** The effect of various glycoconjugates on the growth of intraerythrocytic parasites and the invasion of erythrocytes by merozoites was assessed by growth and invasion inhibition assays. Flat-bottomed 96-well cell culture plates (Costar, Cambridge, Mass.) were first seeded with 140 μl of PRBC suspension, consisting of 7.5% hematocrit and 0.5% early schizont parasites (for the invasion inhibition assay) or 3% ring parasites (for the growth inhibition assay).

Serial dilutions (0, 12.5, 25, 50, 100, and 200 μg/ml) of test reagents in 40 μl of culture medium were then transferred in quadruplicate to the wells. After addition of 20 μl of culture medium containing 1 μCi of [^{3}H]hypoxanthine to each well, the plates were cultured in a sealed container filled with 5% O_2, 5% CO_2, and 90% N_2 for 24 h (growth inhibition assay) or 48 h (invasion inhibition assay). Radioactivities were measured on a Packard Matrix 96 Direct Beta Counter (Packard Instrument Co., Downers Grove, Ill.) after cultures were harvested onto glass fiber filters on a Packard Micromate 196 Harvester (Packard Instrument Co.). The inhibition efficacy of glycoconjugates on parasite growth or invasion was calculated as follows: % inhibition = 100 × [(mean counts per minute [cpm] of control wells − mean cpm of treated wells)/mean cpm of control wells].

The effect of dextran sulfate 500K on the invasion of erythrocytes by *P. falciparum* merozoites was also assessed by the measurement of changes in parasitemia in separate assays.

Cytoadhesion inhibition assays. HMEC-1 and C32 melanoma cells were cultured in endothelial basal medium (Clonetics, Santa Ana, Calif.) and Eagle's minimal essential medium (Gibco), respectively. Binding of PRBC to endothelial cells and C32 melanoma cells was conducted with eight-well tissue culture chamber glass slides (Nunc, Napierville, Ill.) in quadruplicate. Cells (3×10^4) in 0.3 ml of medium were added to each well 24 to 48 h before the adhesion assay. Shortly before the addition of PRBC, the wells were washed twice with RPMI 1640 and replenished with 0.3 ml of binding medium (RPMI 1640 with 10% fetal bovine serum and 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.1]). The test glycoconjugates were added to the lower four wells immediately before the addition of 30 μl of PRBC suspension of 10% hematocrit to all wells. After incubation for 1.5 h at 37°C, the chamber was peeled off and the slide was rinsed with RPMI 1640. The slide was air dried, fixed with methanol, and stained with 3% Giemsa stain. The numbers of PRBC bound to 100 to 500 target cells were counted. Percent binding inhibition was based on the difference between the control and treated wells.

In some binding inhibition assays, PRBC were incubated with glycoconjugates (1 mg/ml) for 30 min and washed twice before they were used. To see whether glycoconjugates would reverse the binding, PRBC were first allowed to bind to target cells for 1 h before test compounds were added. The binding reactions were allowed to continue for another hour. To eliminate the possibility that heparan sulfate proteoglycans were used by PRBC as receptors, HMEC-1 were treated with heparinase (10 U/ml) for 30 min before they were used in some binding assays. Binding of PRBC to immobilized CD36 protein was assayed as described before (21).

Effect of dextran sulfate 500K on the parasitemia and survival of mice infected with *Plasmodium berghei* ANKA. The effect of dextran sulfate 500K on the invasion of erythrocytes by malaria parasites was also evaluated in vivo by treating mice infected with *P. berghei*. Fifty-five 6-week-old female CBA/Ca mice were divided into five groups of 11 animals each. Group 1 was an uninfected control group; animals in this group were uninfected but treated with dextran sulfate 500K subcutaneously twice daily at 200 μg per mouse for 6 days. Group 2 was an infected-untreated group; mice were each inoculated intraperitoneally with 5×10^5 *P. berghei* ANKA PRBC. Group 3 was an infected-treated group; mice in this group were similarly infected but treated twice with dextran sulfate on the day when they were inoculated. Groups 4 and 5 were also infected-treated groups; mice in these two groups, however, were treated with dextran sulfate 500K twice daily for 3 and 6 days, respectively, starting on the day that they were inoculated. Animals in all groups were examined daily for survival and tail-bled on days 4, 5, 7, 8, 11, 18, and 21 for parasitemia determinations.

Statistical analysis. Results from cytoadherence experiments were expressed as means ± standard error of the mean (SEM). Differences between the control and treatment groups were compared by a paired Student's *t* test. Parasitemia levels among different groups in the *P. berghei* experiment were compared by Fisher's protected least significant difference method. Significance was declared at $P \leq 0.05$.

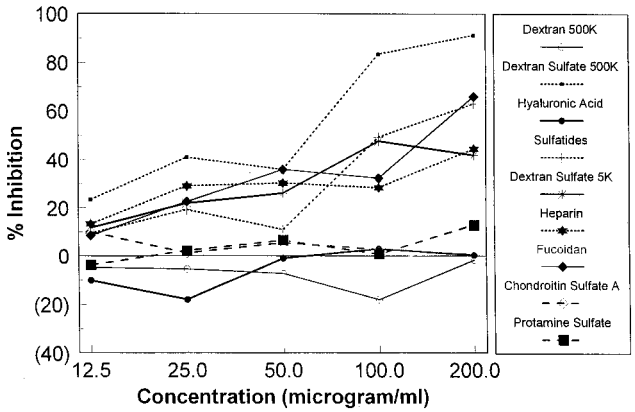

FIG. 1. Effects of various glycoconjugates on the invasion of human O^+ erythrocytes by HB3EC-6 merozoites at different concentrations. Results are from one representative of two experiments conducted in quadruplicate.

RESULTS

Sulfated glycoconjugates inhibit the invasion of erythrocytes by *P. falciparum*. Addition of glycoconjugates to early schizont cultures of HB3EC-6 had a range of effects on the invasion of erythrocytes by merozoites (Fig. 1). Dextran sulfate 500K, dextran sulfate 5K, sulfatides, fucoidan, and heparin all inhibited invasion, with dextran sulfate 500K having the greatest effect. The inhibition was concentration dependent; for most compounds, the effect was low at 12.5 μg/ml, increased at 25, 50, and 100 μg/ml, and highest at 200 μg/ml. Unsulfated dextran 500K and hyaluronic acid had no significant inhibitory effect on invasion. Similarly, sulfated compounds with a weak negative charge (chondroitin sulfate A) or a positive charge (protamine sulfate) did not inhibit invasion. We also examined whether the observed effect of dextran sulfate 500K was due to inhibition of schizont bursting and merozoite release, rendering fewer or no merozoites available for invasion. The study revealed that except for a few ring stage parasites (85 to 93% reduction in parasitemia), no schizonts were seen in erythrocytes 8 or 24 h after dextran sulfate 500K (100 μg/ml) was added to the schizont culture. This result indicates that this sulfated glycoconjugate had no effect on schizont bursting.

Sulfated glycosaminoglycans have no effect on the growth of intraerythrocytic parasites. To eliminate the possibility that the inhibitory effect of glycoconjugates on the invasion was due to the detrimental effect of these compounds on the growth of intraerythrocytic parasites, ring stage parasites were cultured for 24 h in the presence of various concentrations of glycoconjugates. We found that unsulfated and sulfated polysaccharides (fucoidan) and glycosaminoglycans (dextran, dextran sulfate, hyaluronic acid, heparin, and chondroitin sulfate) had no significant effect on the growth of intraerythrocytic parasites (Fig. 2). The positively charged arginine-rich small protein protamine sulfate also had no effect on the growth of parasites. We did, however, observe that the sulfated glycolipid sulfatides inhibited the growth of ring stage parasites in a dose-dependent manner.

Cytoadherence of PRBC can be inhibited by sulfated glycoconjugates. Glycoconjugates had various effects on the binding of HB3EC-6 PRBC to HMEC-1 (Fig. 3 and Table 1). Binding was inhibited by addition of the negatively charged dextran sulfate 500K, fucoidan, and sulfatides, with dextran sulfate 500K having the greatest effect. The differences between the treated and control wells were significant at concentrations of ≥10 μg/ml ($P \leq 0.05$). The inhibitory effect of dextran sulfate

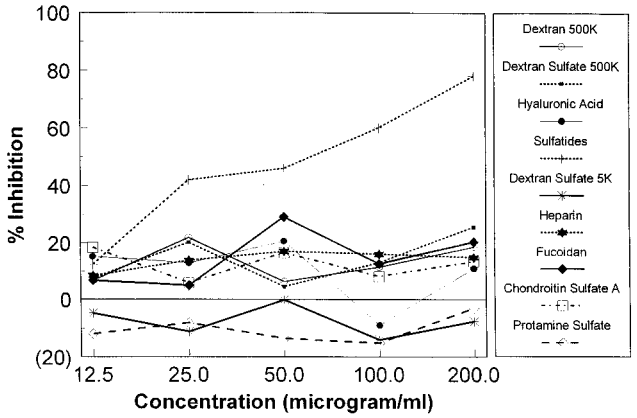

FIG. 2. Effects of various glycoconjugates on the growth of ring stage parasites of *P. falciparum* at different concentrations. Results are from one representative of two experiments conducted in quadruplicate.

500K was also significant at 5 μg/ml. Some other negatively charged compounds, such as heparin (250 μg/ml), heparan sulfate (25 and 250 μg/ml), and dextran sulfate 5K (100 μg/ml), also inhibited binding significantly ($P \leq 0.05$), but the intensity was much less than with dextran sulfate 500K. Compounds with low negative charge, such as keratan sulfate, chondroitin sulfate A, hyaluronic acid, and unsulfated and uncharged dextran 500K, however, had no significant effect on binding ($P \geq 0.05$). The inhibitory effect of sulfated glycoconjugates increased only slightly with the increase in concentrations. To the contrary, the positively charged protamine sulfate promoted binding significantly at 25, 100, 250, and 500 μg/ml ($P \leq 0.05$). These compounds also had similar effects on the binding of HB3C32-6 PRBC to C32 melanoma cells (Table 2).

Sulfated glycoconjugates, however, could not disrupt binding if they were added after the binding between PRBC and target cells had already taken place (Table 3). Although significantly lower binding intensities were usually observed in the group treated with dextran sulfate 500K ($P \leq 0.05$), the effect was probably due to the blockage of further binding during the second hour of incubation rather than disruption of cytoadherence. In control experiments, when we did not add glycoconjugates, binding after 2 h of incubation was normally higher than after 1 h, indicating that some PRBC bound to target cells during the second hour of incubation.

Sulfated glycoconjugates are not receptors for the cytoadherence of PRBC. Because sulfated proteoglycans are used as adhesion receptors by several infectious agents, including plasmodial sporozoites, we investigated whether sulfated glycoconjugates act as receptors for cytoadhesion by PRBC. Preincubation of PRBC with dextran sulfate 500K did not inhibit the binding of HB3EC-6 PRBC to HMEC-1 (Table 3). Although the binding of HB3C32-6 PRBC to C32 melanoma cells was inhibited by 31%, this effect was not significant ($P \geq 0.05$). Pretreatment of PRBC with dextran sulfate inhibited the binding of HB3C32-6 to immobilized CD36 but increased the binding of another parasite line, HB3EC-6, to this protein ($P \leq 0.05$). We believe that this inconsistency in binding inhibition was probably due to differences in the washing process, which was difficult to control when binding assays were conducted with immobilized proteins. Unlike the binding inhibition assays conducted with cell culture on chamber slides, binding with PRBC treated with glycoconjugates or left untreated had to be done in separate dishes. It is possible that the washing conditions between culture dishes were different. It is important to note that this is an exception rather than the rule in this study. Cleavage of heparan sulfate chains (the major sulfated glycoconjugate on endothelial cells) from target cells by heparinase treatment also had no effect on the binding of HB3EC-6 PRBC to HMEC-1 ($P \geq 0.05$, Table 3).

Effect of dextran sulfate 500K treatment on the parasitemia and survival of mice infected with *P. berghei*. Because in vitro studies demonstrate that dextran sulfate 500K inhibits the invasion of erythrocytes by both *P. falciparum* (this study) and *P. knowlesi* (8), we evaluated the efficacy of dextran sulfate 500K treatment on the parasitemia and survival of mice infected with *P. berghei* ANKA, a model for cerebral malaria. In this model, CBA/Ca mice inoculated with *P. berghei* ANKA blood stage parasites die either from cerebral malaria during early infection (6 to 14 days postinoculation) or from severe anemia during late infection (3 to 4 weeks postinoculation) (13). During the cerebral malaria phase of the infection (days 7 to 11), more mice that received dextran sulfate 500K for 3 or 6 days postinfection died than in the groups treated for only 1 day or left untreated (Fig. 4). In the anemia phase of the infection (day 21 and beyond), however, mice treated for 3 or 6 days died later than those left untreated or treated for only 1 day. Mice treated with dextran sulfate 500K for 3 or 6 days postinfection had lower parasitemia than untreated mice throughout the course of infection (Fig. 5). The difference between these two treated groups and the untreated group was significant on days 4, 18, and 21 ($P \leq 0.05$). Parasitemia in the mice treated with dextran sulfate 500K for 1 day was also significantly lower than in the untreated mice on day 4 ($P \leq 0.05$), although subsequently the parasitemia in this group was similar to that in the untreated group.

DISCUSSION

Sulfated glycoconjugates, especially heparan sulfate, have been implicated as receptors in the invasion of several infectious agents, such as human immunodeficiency virus, herpes simplex virus, *Mycoplasma hyopneumoniae*, *Haemophilus influenzae*, and *Trypanosoma cruzi* (11, 16, 20, 22, 35). Two proteins involved in plasmodial sporozoite invasion of hepatocytes, circumsporozoite protein and thrombospondin-related anonymous protein, also use heparan sulfate proteoglycans on hepatocytes as receptors (5, 19, 23). Recently, it has been suggested that sulfated glycoconjugates are also used by other develop-

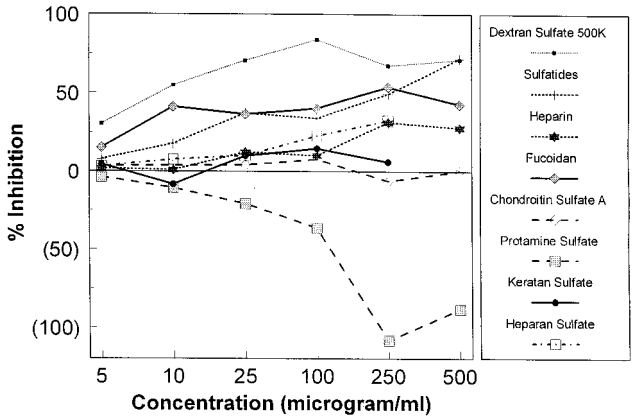

FIG. 3. Binding of HB3EC-6 to HMEC-1 can be inhibited by negatively charged sulfated glycoconjugates and promoted by positively charged protamine sulfate. Compounds were added to HMEC-1 cultures immediately before the addition of PRBC. Percent inhibition was based on experiments done in quadruplicate.

TABLE 1. Effects of sulfated glycoconjugates (100 μg/ml) on the binding of HB3EC-6 PRBC to HMEC-1 cells

Substance	Major component	Mol wt	Sulfation	Charge	Inhibition[a] (%)
Dextran 500K	Glucose	500,000	—	Neutral	−9.1
Dextran sulfate 500K	Glucose	500,000	High	High −	83.9*
Dextran sulfate 5K	Glucose	5,000	High	High −	25.3*
Fucoidan	Fucose	180,000	Low	Low −	40.2*
Sulfatides	Galactose	907	Low	Low −	34.0*
Heparin	Glucuronic acid and acetylglucosamine	3,000–30,000	Medium	Medium −	10.2
Heparan sulfate	Glucuronic acid and acetylglucosamine	7,000–14,000	Medium	Medium −	22.8
Keratan sulfate	Galactose and acetylgalactosamine	109,000	Low	Low −	14.7
Chondroitin sulfate A	Glucuronic acid and acetylgalactosamine	50,000	Low	Low −	7.8
Hyaluronic acid	Glucuronic acid and acetylglucosamine	50,000–8,000,000	—	Low −	−20.3
Protamine sulfate	Basic amino acids	5,000	Low	High +	−36.4*

[a] Negative values denote binding promotion. An asterisk indicates that the difference between the control wells and wells with sulfated glycoconjugates is significant ($P \leq 0.05$).

mental stages of malaria parasites as invasion and cytoadhesion receptors (26–28). The highly sulfated heparin and other sulfated conjugates such as dextran sulfate, fucoidan, and sulfatides are well known for their ability to disrupt *P. falciparum* erythrocyte rosettes (3, 27, 28). These compounds have also been shown to inhibit the growth of intraerythrocytic *P. falciparum* (2). In addition, dextran sulfate and fucoidan can inhibit the invasion of human Duffy-positive and rhesus erythrocytes by *Plasmodium knowlesi* (8), and heparin can inhibit the invasion of human erythrocytes by *P. falciparum* (16). A recent study has indicated that chondroitin sulfate A is a receptor for the cytoadherence of PRBC to endothelial cells (26). From these studies, it has been suggested that sulfated glycoconjugates probably also serve as receptors for invasion, rosette formation, and cytoadherence of PRBC to endothelial cells (26–28).

The results of the present study extended the previous observation that invasion of erythrocytes by *P. falciparum* merozoites could be partially blocked by heparin (16, 32); other sulfated glycoconjugates were also shown to inhibit invasion. In fact, dextran sulfate 500K had a higher inhibitory effect than heparin. The inhibition of invasion by these glycoconjugates was not the result of detrimental effects on the parasites, because most of the compounds (except for the sulfatides) had no effect on the growth of ring stage parasites. This is much different from the results of an earlier study, in which dextran sulfate, fucoidan, heparin, and some other sulfated glycoconjugates were shown to inhibit the growth of ring stage parasites (2). The inhibitory effect of sulfatides on the growth of ring stage parasites observed here is probably the result of entrance of this glycolipid into erythrocytes because of its small size and lipophilic nature. The inhibition potency of heparin on invasion seen in this study is much smaller than previously reported (16, 32).

The binding of PRBC to endothelial cells can also be inhibited by negatively charged sulfated glycoconjugates. Like their effects on invasion, compounds with high negative charge (dextran sulfate 500K) had a higher inhibitory effect on both invasion and cytoadherence than those with moderate negative charge (heparin and heparan sulfate). Most glycoconjugates with weak negative charge (chondroitin sulfate A, keratan sulfate, and hyaluronic acid) had no effect. Dextran sulfate 5K, a constituent fragment of dextran sulfate 500K, had a much lower effect than dextran sulfate 500K. Likewise, although fucoidan has a low negative charge, it inhibited invasion and cytoadherence moderately, probably because of its large size. Factors beyond charge intensities and molecular sizes, however, were probably responsible for the failure of keratan sulfate and hyaluronic acid to inhibit cytoadherence. The carbohydrate moiety of the glycoconjugate is probably not important for the inhibition of invasion and cytoadherence. For example, the carbohydrate components in heparin and heparan sulfate are similar to those in the unsulfated hyaluronic acid, but only heparin and heparan sulfate inhibited invasion and cytoadherence. Unlike the negatively charged sulfated glycosaminoglycans, the positively charged protamine sulfate promoted the binding of PRBC to endothelial cells but not invasion. Chondroitin sulfate A was apparently not used by the *P. falciparum* isolates used in this study as receptors for PRBC binding to

TABLE 2. Effects of sulfated glycoconjugates on the binding of HB3C32-6 PRBC to C32 melanoma cells

Substance	Mean no. of PRBC bound/C32 cell ± SEM[a] (% inhibition)			
	Control	Treated (25 μg/ml)	Control	Treated (100 μg/ml)
Dextran sulfate 500K	492.5 ± 12.4	192.0 ± 13.4 (61.4)*	189.5 ± 19.5	21.5 ± 3.9 (88.7)*
Dextran sulfate 5K	444.3 ± 50.8	270.0 ± 29.3 (39.2)*	772.0 ± 47.2	282.8 ± 49.6 (63.4)*
Fucoidan	471.3 ± 66.9	222.0 ± 24.9 (52.9)*	232.5 ± 26.3	87.5 ± 9.1 (62.4)*
Sulfatides	504.3 ± 22.9	241.3 ± 27.7 (52.2)*	349.5 ± 22.1	166.0 ± 21.5 (52.5)*
Heparin	395.3 ± 17.3	245.5 ± 21.0 (37.9)*	332.3 ± 17.3	241.5 ± 35.0 (27.3)
Heparan sulfate	410.3 ± 15.1	315.8 ± 17.0 (23.0)	196.8 ± 22.9	156.3 ± 6.4 (20.6)
Keratan sulfate	426.5 ± 26.3	418.8 ± 34.0 (1.8)	190.3 ± 20.2	205.5 ± 33.6 (−8.0)
Chondroitin sulfate A	426.8 ± 13.3	431.5 ± 24.1 (−1.1)	298.8 ± 9.1	259.8 ± 14.2 (13.1)
Protamine sulfate	325.0 ± 17.6	414.8 ± 46.4 (−27.6)	294.5 ± 10.7	467.8 ± 42.0 (−58.8)*

[a] C32 cells were treated with sulfated glycoconjugates at 25 or 100 μg/ml or left untreated (control). Numbers in parentheses show percent inhibition (positive values) or promotion (negative values) of binding after addition of sulfated glycoconjugates. An asterisk indicates that the difference between the control wells and wells treated with sulfated glycoconjugates is significant ($P \leq 0.05$).

TABLE 3. Sulfated glycoconjugates are not used directly as receptors for the binding of HB3EC-6 to endothelial cells and HB3C32-6 to melanoma cells

Expt[a]	PRBC	Target cells	Substance	Dose	% Inhibition of binding[b]
Disruption of cytoadherence	HB3EC-6	HMEC-1	Dextran sulfate 500K	100 μg/ml	27.0*
			Heparin	100 μg/ml	2.8
	HB3C32-6	C32 melanoma	Dextran sulfate 500K	100 μg/ml	26.0*
			Heparin	100 μg/ml	11.2
Pretreatment of PRBC with glycoconjugates	HB3EC-6	HMEC-1	Dextran sulfate 500K	1 mg/ml	6.9
	HB3C32-6	C32 melanoma	Dextran sulfate 500K	1 mg/ml	31.0
	HB3EC-6	CD36	Dextran sulfate 500K	1 mg/ml	−59.7*
	HB3C32-6	CD36	Dextran sulfate 500K	1 mg/ml	23.2*
Pretreatment of target cells	HB3EC-6	HMEC-1	Heparinase	10 U/ml	2.9

[a] In the first experiment, binding was assessed 2 h after addition of PRBC and 1 h after the addition of glycoconjugates. In the second, PRBC were incubated with dextran sulfate 500K for 30 min at 37°C and washed twice before they were used in binding assays. In the third, HMEC-1 cells were treated with heparinase at 37°C for 2 h before the binding assay.

[b] Negative values denote binding promotion. The asterisk indicates that the difference between the control wells and wells with sulfated glycoconjugates is significant ($P \leq 0.05$).

endothelial cells, because chondroitin sulfate A did not inhibit cytoadherence even when it was used at 500 μg/ml.

The inhibitory effect of sulfated glycoconjugates on the invasion, rosetting, and cytoadherence of PRBC can be simply a result of nonspecific electrostatic interactions rather than a consequence of specific cytoadhesion receptors. All the glycoconjugates used in previous studies were negatively charged. Because merozoites (phospholipids), erythrocytes (sialic acid in glycophorins and glycolipids), and endothelial cells (sialic acid and sulfated proteoglycans) all have net negative charges (29, 34), it is possible that the negatively charged sulfated glycoconjugates exert their effect on the invasion, rosetting, and sequestration of malaria parasites by increasing the charge repulsive force between cells or sterically interfering with the binding of specific ligands to receptors. The failure of preincubation of PRBC with sulfated glycoconjugates and the cleavage of heparan sulfate chains on endothelial cells to inhibit cytoadherence further supports the theory that sulfated glycoconjugates were not used by endothelial cells directly as binding receptors.

Because the inhibitory effect of sulfated glycoconjugates on erythrocyte invasion by merozoites seems to be a feature common to all malaria parasites tested (*P. falciparum* and *P. knowlesi*), we evaluated the in vivo effect of dextran sulfate 500K on the invasion of erythrocytes by *P. berghei* merozoites. Infected mice treated with dextran sulfate 500K for 3 or 6 days had lower parasitemia during the entire course of infection than those left untreated or treated for only 1 day. We also found that these treated mice died later during the anemia phase of the infection. This effect can be explained by the reduced parasitemia due to treatment. In contrast, we observed that a higher number of treated mice died during the cerebral malaria phase of the infection. The latter result was probably due to the increased occurrence of brain hemorrhage as a result of treatments with dextran sulfate. Brain hemorrhage is a general feature of both murine and human malaria (7, 30). Although it is much less potent than heparin (17 USP units/mg versus up to 170 USP units/mg), dextran sulfate 500K nevertheless is an anticoagulant. Previously, heparin was used as an ancillary treatment for human cerebral malaria in the belief that disseminated intravascular coagulation is important in the pathogenesis of cerebral malaria, a view no longer held

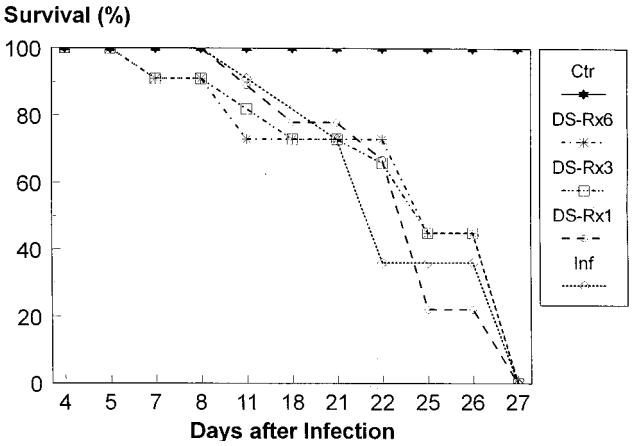

FIG. 4. Effect of dextran sulfate 500K treatments on the survival of CBA/Ca mice infected with *P. berghei* ANKA. Groups of 11 mice each were treated with dextran sulfate 500K (twice daily at 200 μg per mouse subcutaneously) for 1 (DS-Rx1), 3 (DS-Rx3), or 6 (DS-Rx6) days, starting on the day when they were inoculated (5×10^5 PRBC per mouse intravenously). One group of mice (Ctr) were not inoculated but were treated with dextran sulfate 500K for 6 days. Another group of mice (Inf) were inoculated but untreated.

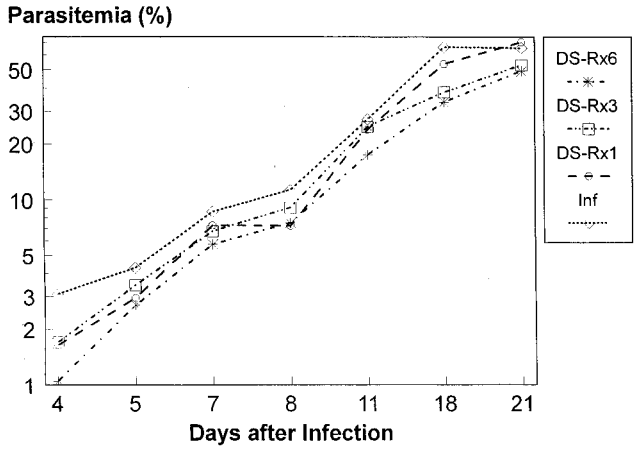

FIG. 5. Effect of dextran sulfate 500K treatments on the parasitemia of CBA/Ca mice infected with *P. berghei* ANKA. Groups of mice were treated with dextran sulfate 500K for 1 (DS-Rx1), 3 (DS-Rx3), or 6 (DS-Rx6) days, starting on the day when they were infected. One group of mice (Ctr) were not inoculated but were treated with dextran sulfate 500K for 6 days. Another group of mice (Inf) were inoculated but untreated. Mice in the Ctr group had no parasitemia during the course of the experiment.

by most researchers. Although one study with simian malaria found that heparin treatment reduced parasitemia and mortality (9), subsequent studies with monkeys and humans showed no beneficial effect of heparin treatment (14, 25).

In conclusion, invasion of erythrocytes by merozoites and cytoadherence of PRBC to endothelial cells can be inhibited by negatively charged glycoconjugates. These compounds possibly inhibit both processes (invasion and cytoadherence) by increasing the charge repulsive force between cells or steric interference with the ligand-receptor interaction. New intervention strategies based on the effect of glycoconjugates can be developed to reduce both the invasion of erythrocytes by merozoites and sequestration of PRBC, which in turn limits the occurrence of severe anemia and cerebral malaria without interfering with the development of natural immunity.

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