Malaria Sporozoites and Circumsporozoite Proteins Bind Specifically to Sulfated Glycoconjugates

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Abstract. Circumsporozoite (CS) proteins, which densely coat malaria (*Plasmodia*) sporozoites, contain an amino acid sequence that is homologous to segments in other proteins which bind specifically to sulfated glycoconjugates. The presence of this homology suggests that sporozoites and CS proteins may also bind sulfated glycoconjugates. To test this hypothesis, recombinant *P. yoelii* CS protein was examined for binding to sulfated glycoconjugate-Sepharoses. CS protein bound avidly to heparin-, fucoidan-, and dextran sulfate-Sepharose, but bound comparatively poorly to chondroitin sulfate A- or C-Sepharose. CS protein also bound with significantly lower affinity to a heparan sulfate biosynthesis-deficient mutant cell line compared with the wild-type line, consistent with the possibility that the protein also binds to sulfated glycoconjugates on the surfaces of cells. This possibility is consistent with the observation that CS protein binding to hepatocytes, cells invaded by sporozoites during the primary stage of malaria infection, was inhibited by fucoidan, pentosan polysulfate, and heparin.

The effects of sulfated glycoconjugates on sporozoite infectivity were also determined. *P. berghei* sporozoites bound specifically to sulfatide (galactosyl[3-sulfate]β1-1ceramide), but not to comparable levels of cholesterol-3-sulfate, or several examples of neutral glycosphingolipids, gangliosides, or phospholipids. Sporozoite invasion into hepatocytes was inhibited by fucoidan, heparin, and dextran sulfate, paralleling the observed binding of CS protein to the corresponding Sepharose derivatives. These sulfated glycoconjugates blocked invasion by inhibiting an event occurring within 3 h of combining sporozoites and hepatocytes. Sporozoite infectivity in mice was significantly inhibited by dextran sulfate 500,000 and fucoidan. Taken together, these data indicate that CS proteins bind selectively to certain sulfated glycoconjugates, that sporozoite infectivity can be inhibited by such compounds, and that invasion of host hepatocytes by sporozoites may involve interactions with these types of compounds.

To initiate an infection, malaria (*Plasmodium* sp.) sporozoites must pass from the salivary gland of an infected Anopheles mosquito into the host's blood stream during feeding, followed by invasion of host hepatocytes. Little is known of the mechanisms used by sporozoites to arrive at their desired destination and then to selectively bind to and invade host hepatocytes. Circumsporozoite (CS)[1] proteins densely coat sporozoites, and these proteins

apparently play a critical role in the invasion of sporozoites into hepatocytes since some antibodies against such proteins inhibit invasion of hepatocytes and the cell surface expression of CS proteins correlates with sporozoite infectivity (for review see reference 13). The sequences of all CS proteins reported to date contain the amino acid homology Cys-Ser-Val-Thr-Cys-Gly-*x*-Gly-*x*-*x*-*x*-Arg-*x*-Arg/Lys (see references in 7). Thus, this sequence, located in the carboxyl-terminal of the protein referred to as region II, may encode a crucial CS protein functional domain. This amino acid homology also occurs in the recently described sporozoite surface protein 2 (6), a minor component of the cell surface proteins on sporozoites, further suggesting that this domain plays an important role for the malaria parasite.

Interestingly, thrombospondin, von Willebrand factor, collagen Type IV, β-2 glycoprotein I, antistasin, and properdin also possess one or more examples of the amino acid homology sequence described above (7). Although these proteins are involved in widely diverse biological processes, they all specifically bind sulfated glycoconjugates (8). For

1. *Abbreviation used in this paper:* CS, circumsporozoite.

example, where examined in detail these proteins have been observed to bind with comparatively high affinity to sulfated glycoconjugates such as heparin, fucoidan, and dextran sulfates, but only weakly to chondroitin sulfates (7, 8, and references therein). Therefore, it has been hypothesized that the amino acid homology segment may be a sulfated glycoconjugate-binding domain (8).

The potential of malaria sporozoites and CS proteins to specifically interact with sulfated glycoconjugates is examined in this report. First, we investigated CS protein binding to sulfated glycoconjugate-Sepharoses. We also examined CS protein binding to heparan sulfate biosynthesis-deficient mutant and wild-type CHO cells, as well as to murine hepatocytes. The effects of sulfated glycoconjugates on infectious sporozoites were also investigated. Sporozoite binding to surfaces coated with various glycolipids and other test compounds was determined. A battery of sulfated glycoconjugates was evaluated for their potency to inhibit the infection of hepatocytes by sporozoites, and the kinetics of inhibition is examined. Finally, the ability of sulfated glycoconjugates to inhibit malaria sporozoite infectivity in vivo was evaluated.

Materials and Methods

Materials

Glycosphingolipids were purchased from Sigma Chem. Co. (St. Louis, MO), Supelco Inc. (Bellefonte, PA), or Bachem California (Torrance, CA). Heparin-Sepharose was from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Heparin, hyaluronic acid, and chondroitin sulfates A and C were from Calbiochem-Behring Corp., San Diego, CA. Fatty acid-free BSA, phospholipids, pentosan polysulfate, chondroitin sulfate B, cholesterol-3-sulfate, dextran sulfates 5,000 and 500,000, and fucoidan were from Sigma Chem. Co. Human thrombospondin was purified as described (7). Immuno-affinity-purified, full-length recombinant *P. yoelii* CS protein, expressed in *Salmonella typhimurium* (21), was produced by Drs. W. Weiss and L. Yuan (Naval Medical Research Institute, Bethesda, MD) from transformed *Salmonella typhimurium* provided by Dr. A. Aggarwal (Walter Reed Army Institute of Research, Washington, DC). ANKA clone *P. berghei* sporozoites were prepared from infected *Anopheles stephensii* mosquitoes using the method of Mellouk et al. (12).

Preparation of Modified Sepharoses

Sulfated glycoconjugates were coupled to divinylsulfone-activated Sepharose 6B by the method of Porath (15). The amounts of glycoconjugate coupled to Sepharose, determined by phenol/sulfuric acid assays (17), were as follows: fucoidan, 0.87 mg/ml; dextran sulfate 5,000, 2.09 mg/ml; dextran sulfate 500,000, 1.81 mg/ml; chondroitin sulfate A, 1.74 mg/ml; chondroitin sulfate C, 0.93 mg/ml.

CS Protein Binding Assays to Modified Sepharoses

CS protein or thrombospondin were radiolabeled with ^{125}I (ICN Biomedicals, Inc., Costa Mesa, CA) as described (19), except that unincorporated ^{125}I was removed on columns of Bio-Gel P10 (100–200 mesh, Bio-Rad Labs.-Chem. Div., Richmond, CA). The following manipulations were done at room temperature. Triplicate columns of modified Sepharose (150 μl) were preblocked by washing extensively with 50 mM Tris, pH 7.8, 120 mM NaCl, 2.5 mM CaCl$_2$, 1% BSA. The columns were next equilibrated in 50 mM Tris, pH 7.8, 2.5 mM CaCl$_2$ (buffer A) and ^{125}I-proteins (40–800 fmol) were applied in buffer A containing 30 mM NaCl, 0.25% BSA. Columns contained at least 100-fold molar excess of derivatized sulfated glycoconjugates compared with the radiolabeled samples added. The columns were washed with buffer A (3 × 1 ml), and then eluted successively with 1 ml each of buffer A containing 0.15, 0.3, 0.45, or 0.6 M NaCl. Radiolabeled material eluting in each fraction was determined by γ-counting. ^{125}I-Proteins eluting in buffer A containing 0.15-M NaCl concentrations or higher were included in calculations of the protein bound to a given column. Calculations of moles bound were made by first correcting for

binding to unmodified Sepharose, which was 11 and 13% of maximal binding observed to modified Sepharoses for ^{125}I-thrombospondin and ^{125}I-CS protein, respectively. Thrombospondin had been affinity purified on a heparin-Sepharose column before iodination (7). Iodination produced a major loss of heparin binding activity, resulting in only ~20% of ^{125}I-thrombospondin binding to heparin-Sepharose. The extent of loss of CS protein binding activity after iodination is unknown since this cloned gene product was of uncertain conformational state and, although it had been immunoaffinity purified before labeling, had not been affinity purified on heparin-Sepharose.

CS Protein Binding to Cells

Wild-type (K1) or heparan sulfate biosynthesis-deficient (*pgsA* 803, ~90% reduced heparan sulfate and ~50% reduced chondroitin sulfate biosynthesis) CHO cell lines (5) were generously provided by Dr. Jeffrey Esko (University of Alabama at Birmingham). Trypsinized cells (400 μl, 2 × 10^5 cells/ml in Ham's F12 media, 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin) were aliquoted in the wells of Cluster^{24} plates (Costar Corp., Cambridge, MA). The cells were cultured 18 h at 37°C in a 5% CO$_2$ atmosphere, reconstituting their surfaces and achieving ~70% confluence. The wells were gently washed three times with ice-cold buffer A containing 1% BSA, 0.15 M NaCl. Dilutions of ^{125}I-proteins in the same buffer were aliquoted into duplicate wells (≪1 pmol/well at highest concentration; diluted 1:2 thereafter), and the plates were incubated 3 h at 4°C. Unbound material was removed from the plate with five washes of ice-cold PBS, and the bound ^{125}I-proteins were quantitated by γ-counting and corrected for binding to wells without cells. Microscopic inspection and trypan blue dye exclusion of wells treated in parallel indicated that cell viability remained >90% in these experiments.

For assays of the inhibitory potency of sulfated glycoconjugates on CS protein binding to hepatocytes, hepatocytes were prepared from collagenase-treated BALB/c mouse livers as described (12). Hepatocytes were aliquoted into the wells of Cluster^{24} plates (1 ml, 2 × 10^5 cells/ml in MEM supplemented as described in reference 12), and incubated 18 h at 37°C in a 5% CO$_2$ atmosphere, which gave ~70% confluence. The wells were gently washed three times with ice-cold buffer A containing 1% BSA, 0.15 M NaCl. Triplicate samples of ^{125}I-proteins (≪2.0 pmol in 250 μl) containing inhibitors at 400 μg/ml in the same buffer were aliquoted into wells, and the plates incubated 2 h at 4°C. Unbound material was removed from the plate with four washes of ice-cold buffer A containing 1% BSA, 0.15 M NaCl. Bound ^{125}I-proteins were quantitated by γ-counting. Inhibitors had no effect on cell viability, as judged by the lack of an apparent change in cell confluence in their presence.

Sporozoite Binding on Lipid-coated Glass Slides

Test materials were diluted in phosphatidylcholine and cholesterol (200 ng/ml each in methanol). 5-μl aliquots were air dried in the wells of HTC (heavy teflon coated) supercured glass slides (Cel-Line Assoc., Newfield, NJ), and the slides were blocked by incubation in buffer A containing 1% BSA, 0.15 M NaCl for 18 h at 37°C. The blocked slides were rinsed with distilled water and air dried. Freshly isolated sporozoites (10 μl, 3 × 10^5 per ml in RPMI-1640, 5% FCS, 10 mM Hepes, pH 7.4) were overlaid in the slide wells, and binding proceeded 3 h at 4°C. Excess cells were washed from the slides with PBS. Sporozoite binding was determined blindly by immunofluorescence microscopy with anti-*P. berghei* CS protein repeat region mAb, NBS1 (kindly provided by Dr. Y. Charoenvit, Naval Medical Research Institute, Bethesda, MD) and fluoresceinated rabbit anti-mouse IgG antisera (Miles-Yeda Inc., Elkhart, IN).

Sporozoite Infection Assays with Hepatocytes

Hepatocytes were prepared as above, and 10^5 cells were plated in chamber slide plates (Lab-Tek Div. Miles Laboratories Inc., Naperville, IL) overnight. Freshly isolated sporozoites (12) and inhibitor were combined in RPMI-1640, containing 5% FCS and 10 mM Hepes, and 75 μl (2–4 × 10^4 sporozoites) was added to triplicate wells. After an incubation of 3 h at 37°C, unbound sporozoites were gently rinsed from the plates with supplemented MEM. The plates were incubated for an additional 45 h, replacing the media at 24 h, and then fixed and stained for schizonts using an indirect fluorescent antibody test (12). To determine if the inhibitory activity of sulfated glycoconjugates required their interaction with extracellular sporozoites, inhibitors were added to hepatocytes for 3 h before sporozoite addition and then rinsed away, or for 3 h with sporozoites, or for 21 h after the 3-h incubation with sporozoites. It should be noted that sporozoites typically complete invasion of hepatocytes with 3 h of incubation together. In

all experiments, inhibition was calculated relative to the decrease in binding compared with uninhibited (PBS) controls. Uninhibited controls averaged 513 schizonts/well over eight independent experiments, with deviations within triplicates (σ_{n-1}) averaging 9.7% of the number of schizonts detected in each experiment. Inhibitors had no effect on hepatocyte viability, as judged by a lack of detectable change in general morphology or substrate attachment. Inhibitors also had no apparent effect on sporozoite viability, judged by no detectable changes in the parasite's morphology, trypan blue dye exclusion, or motility.

Sporozoite Infection Assays with Mice

Sporozoites were combined with inhibitor (1 mg/ml) for 15 min at 0°C. The mixtures (0.1 ml, containing 800–2,000 sporozoites, depending on the trial) were injected into the tail veins of ~8-wk-old female BALB/c mice. Giemsa-stained blood smears were examined for parasites for 21 d, or until the animal became positive for parasitemia.

Results

CS Protein Binding to Sulfated Glycoconjugate-Sepharoses

The possibility that CS proteins bind specifically to sulfated glycoconjugates, suggested by the occurrence of a putative sulfated glycoconjugate-binding domain in the protein's sequence, was examined by several approaches. In one, recombinant *P. yoelii* CS protein binding to sulfated glycoconjugate-Sepharoses was determined. The CS protein bound extensively to dextran sulfate 500,000– and heparin-Sepharose, and to a lesser extent to fucoidan- and dextran sulfate 5,000–Sepharose (Fig. 1). The binding of the CS protein to chondroitin sulfate A– or C–Sepharose was low; only slightly more CS protein bound to these resins than to unmodified Sepharose. Since the charge densities of chondroitin sulfates A and C are similar to fucoidan, heparin, and dextran sulfates (2), the differences observed in CS protein binding to the sulfated glycoconjugate-Sepharoses is not simply the result of nonspecific charge interactions.

Human thrombospondin, which contains three copies of the homology sequence described above, has been extensively characterized as a sulfated glycoconjugate-binding protein (see references 7, 8, 19, and references therein). Therefore, the binding pattern of thrombospondin on the sulfated glycoconjugate-Sepharoses was determined for comparison with the pattern observed for CS protein. The order of binding preferences for thrombospondin to the modified Sepharoses was remarkably similar to that of the CS protein and the selectivity of binding of CS protein to the dextran sulfates, fucoidan, and heparin vs. the chondroitin sulfates was as good or better than that observed for thrombospondin (Fig. 1). It should also be noted that CS protein and thrombospondin both bind to the modified Sepharoses with orders of preference similar to the sulfated glycoconjugate-binding affinities reported for antistasin and properdin, both of which also contain the homology sequence described above (8). This binding is clearly different from that of such compounds as BSA, which was present in large excess during the binding of CS protein and thrombospondin. Also, binding of iodinated BSA to columns of derivatized Sepharoses under these conditions was <0.2 mol/mol CS protein bound.
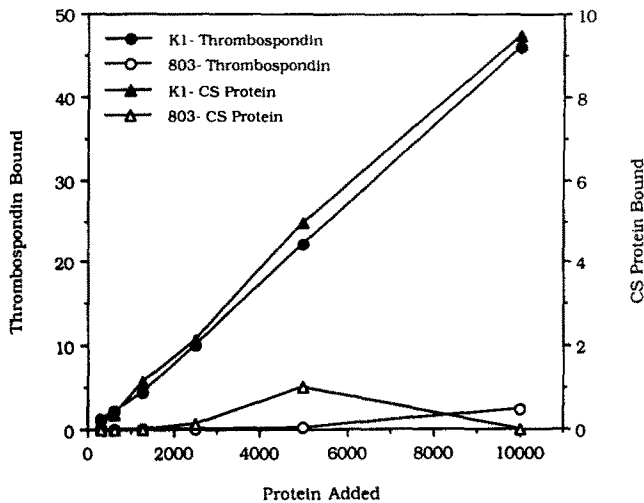
CS Protein Binding to Cell Surfaces

CS protein was also examined for binding to sulfated glycoconjugates in the less artificial context of cell surfaces.

Thrombospondin Bound (fmol)
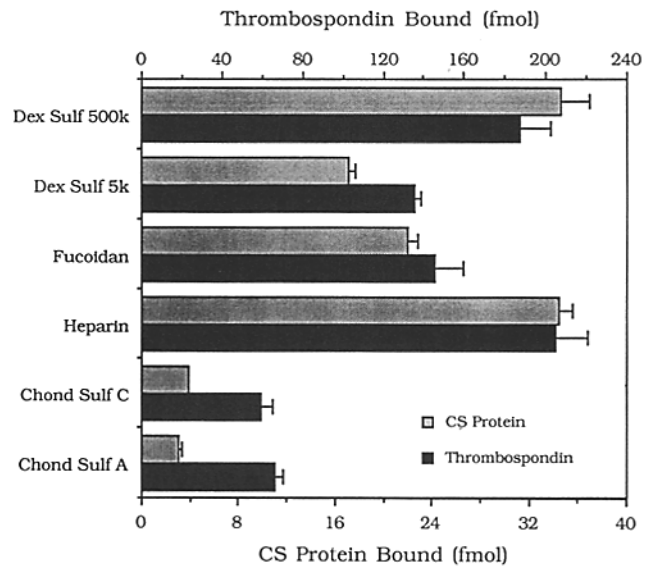
Figure 1. CS protein and thrombospondin bind specifically to sulfated glycoconjugate-Sepharose. ^{125}I-CS protein or ^{125}I-thrombospondin was passed over columns of modified Sepharose and eluted as described in Materials and Methods. ^{125}I-Protein binding to modified Sepharoses corrected for binding to unmodified Sepharose is shown. Data are normalized to binding per pmol thrombospondin of CS protein added. *Dex Sulf 500k* and *Dex Sulf 5k*, dextran sulfate 500,000 and 5,000, respectively; *Chond Sulf A* and *Chond Sulf C*, chondroitin sulfates A and C, respectively. Error bars represent SD (σ_{n-1}).
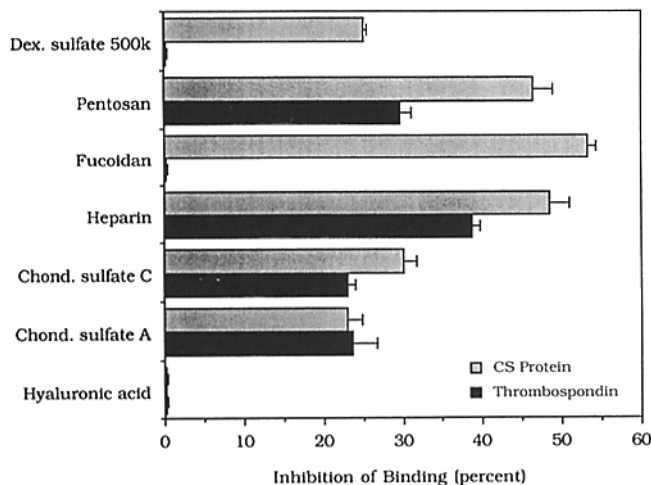
Recently, a cell line (*pgs*A 803) with a mutation in heparan sulfate biosynthesis, resulting in a 90% reduction of cell surface heparan sulfates relative to the parental (K1) line, was described by Esko et al. (4). As shown in Fig. 2, thrombospondin binding to the heparan sulfate biosynthesis-deficient cells was greatly diminished compared with the binding to wild-type cells. These observations are consistent with previous reports suggesting that thrombospondin can interact with the surfaces of various cell types via sulfated glycoconjugates such as heparan sulfate (for review see reference 5). CS protein binding to the heparan sulfate biosynthesis-deficient cells was also greatly reduced compared with that seen to the wild-type cells (Fig. 2), suggesting that this protein can also bind cell surface heparan sulfates.

The potency of sulfated glycoconjugates to inhibit the binding of CS protein to the surfaces of murine hepatocytes, cells invaded by sporozoites during the primary stage of malaria infection, was also determined. CS protein binding to hepatocytes was inhibited by fucoidan, heparin, and pentosan polysulfate (Fig. 3). The activity of fucoidan and heparin is consistent with the binding preferences of CS protein observed for the corresponding modified Sepharoses (Fig. 1). Dextran sulfate 500,000 and the chondroitin sulfates all showed similar, moderate levels of inhibitory activity, in contrast to binding to their Sepharose derivatives, while hyaluronic acid demonstrated none. Thrombospondin binding to hepatocytes was best inhibited by heparin and pentosan polysulfate, moderately by the chondroitin sulfates, and not at all by dextran sulfate 500,000 or fucoidan. This pattern of inhibitory activity clearly differed from the binding activi-
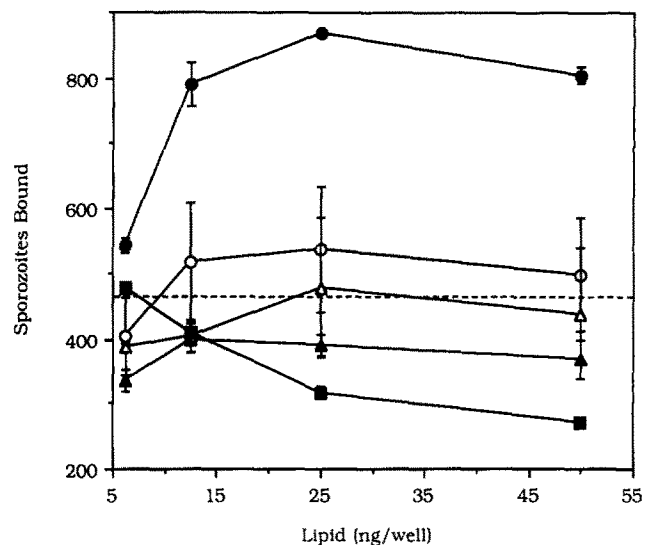
Figure 2. A mutation in the biosynthesis of heparan sulfate decreases the binding of CS protein to cell surfaces. ^{125}I-CS protein or ^{125}I-thrombospondin was incubated with heparan sulfate biosynthesis-deficient mutant (803) or wild-type (*K1*) cell lines adhering to the bottoms of microtiter wells. After an incubation of 3 h at 4°C, the wells were washed and the bound protein was quantitated. Data are expressed as fmoles ^{125}I-CS protein (*CS*), or [125]thrombospondin bound (y axis) vs. fmol added (x axis). The average of the SD (σ_{n-1}) for these data is 5.5% of the average moles bound.

Figure 4. Sporozoites bind specifically to sulfatide. Live *P. berghei* sporozoites were overlaid on glass slides coated with varying amounts of the indicated material. After an incubation of 3 h at 4°C, excess cells were removed and bound cells were quantitated by immunofluorescence microscopy. (●) Sulfatide; (○) cholesterol-3-sulfate; (▲) GM$_1$; (△) phosphatidylserine; (■) galactosylceramide; (---) phosphatidylcholine/cholesterol. Error bars represent SD (σ_{n-1}).

ties observed for the corresponding Sepharose derivatives. One explanation for this observation is that thrombospondin has at least one other sulfated glycoconjugate-binding domain distinct from the one encoded by the amino acid homology sequence described above, presumably with a somewhat different specificity, as well as two additional types of bind-

ing sites, an RGD binding site and a platelet/cell binding site (for review see reference 16). That neither CS protein nor thrombospondin binding is inhibited by hyaluronic acid, a highly carboxylated, high molecular weight carbohydrate polymer that does not contain sulfate moieties, as well as the wide range of inhibitory activities demonstrated by the various sulfate containing glycoconjugates, suggests that the binding of these proteins to hepatocytes is not primarily the result of nonspecific charge effects.

Sporozoite Binding to Glycolipid-coated Surfaces

CS proteins densely coat the surfaces of *Plasmodium* sporozoites (13). Therefore, it was suggested by the CS protein binding data presented above that sporozoites may also bind specifically to sulfated glycoconjugates. In one set of experiments designed to evaluate this possibility, *P. berghei* sporozoites were examined for binding to test materials immobilized on glass slides. As shown in Fig. 4, sporozoites bind in comparatively high numbers to slides coated with the sulfated glycolipid, sulfatide (galactosyl [3-sulfate]β1–lceramide). In contrast, sporozoites bind at, or below, background levels to slides coated with cholesterol-3-sulfate, phosphatidylserine, GM^1, or GM$_2$ (Fig. 4; GM$_2$, data not shown), suggesting that sporozoites binding to sulfatide is not due to nonspecific charge interactions. Sporozoites also do not bind selectively to galactosylceramide (Galβ1–lceramide)–coated surfaces, indicating that a sulfate moiety must be present on the glycoconjugate portion of this glycolipid for binding.

Figure 3. Inhibition of CS protein binding to hepatocytes by sulfated glycoconjugates. ^{125}I-CS protein or ^{125}I-thrombospondin were incubated in the presence of the indicated inhibitors with murine hepatocytes adhering to the bottoms of microtiter wells. After an incubation of 2 h at 4°C, the wells were washed and the bound protein was quantitated. Percent inhibitions are calculated relative to the binding observed for controls in which no inhibitor was added. *Dex. sulfate 500k*, *Pentosan*, *Chond. sulfate A*, and *Chond. sulfate C*, dextran sulfate 500,000, pentosan polysulfate, chondroitin sulfate A, and chondroitin sulfate C, respectively.

Inhibition of Sporozoite Infection of Hepatocytes by Sulfated Glycoconjugates

Sulfated glycoconjugates were assayed for their relative abil-

Table I. Inhibition of Sporozoite Infectivity in Hepatocytes by Sulfated Glycoconjugates*

Inhibitors§	Inhibition (%)‡		
	Trial 1	Trial 2	Trial 3
Dextran sulfate 500,000			
400 µg/ml	–	89	–
Dextran sulfate 5,000			
1,000 µg/ml	94	–	–
400 µg/ml	–	78	63
Fucoidan			
1,000 µg/ml	86	–	–
400 µg/ml	–	54	58
Heparin			
1,000 µg/ml	53	–	–
400 µg/ml	–	72	70
Chondroitin sulfate A			
2,000 µg/ml	–	–	6
400 µg/ml	–	9	0
Chondroitin sulfate B			
400 µg/ml	–	0	–
Chondroitin sulfate C			
2,000 µg/ml	–	–	22
400 µg/ml	–	25	5
Hyaluronic acid			
400 µg/ml	–	20	–

* Sporozoites were overlaid 3 h on hepatocytes bound to slides, the slides were rinsed, and schizonts developing after 48 h were counted.
‡ Inhibition relative to PBS added alone. The total number of schizonts developing in triplicate wells in the presence of PBS alone were 906, 2,751, and 1,883 for trials 1, 2, and 3, respectively.
§ Inhibitors were present only during the initial 3-h incubation.

Table II. Time Course of Inhibition of Sporozoite Infectivity in Hepatocytes by Dextran Sulfate 5,000*

Time inhibitor added§	Inhibition‡		
	Experiment 1	Experiment 2	Experiment 3
	%	%	%
−3 to 0 h			
2 mg/ml	–	26 ± 5	–
1 mg/ml	2 ± 11	–	–
0 to 3 h			
1 mg/ml	58 ± 6	73 ± 9	97 ± 3
0.2 mg/ml	–	–	91 ± 3
3 to 24 h			
1 mg/ml	–	0 ± 11	–
0.2 mg/ml	–	–	0.24

* Sporozoites were overlaid 3 h on hepatocytes bound to slides, the slides were rinsed, and schizonts developing after 48 h counted.
‡ Inhibition relative to PBS added alone.
§ 0 h refers to the time when sporozoites were first overlaid on the hepatocytes.

tivity by a mechanism that requires contact between these inhibitors and extracellular sporozoites.

Inhibition of Sporozoite Infection of Mice by Sulfated Glycoconjugates

The possibility that sulfated glycoconjugates are inhibitory to sporozoite infectivity in in vivo assays, as they are in the in vitro assays presented above, was also investigated. Mice were injected with sporozoites in combination with various inhibitors, and the mice were examined for parasitemia. Fucoidan and dextran sulfate 500,000 clearly inhibited sporozoite infectivity in vivo (Table III), paralleling the observation that these compounds were also among the most effective reagents tested in blocking the in vitro invasion of hepatocytes by sporozoites (Table I). Heparin, dextran sulfate 5,000, chondroitin sulfate C, and hyaluronic acid did not clearly inhibit the ability of sporozoites to infect mice, indicating a highly selective pattern for in vivo inhibitory activity and that the inhibitory effects of fucoidan and dextran sulfate 500,000 are not the result of nonspecific charge effects.

ities to inhibit the infection of mouse hepatocytes by P. berghei sporozoites. P. berghei sporozoites were used rather than P. yoelii sporozoites because of their greater infectivity for mouse hepatocytes in vitro. In addition, P. berghei and P. yoelii CS proteins differ at only 2 amino acids out of 16 in the region of homology (consensus region) described above (20). Dextran sulfates 5,000 and 500,000, fucoidan, and heparin were found to be good inhibitors of hepatocyte infection (Table I), consistent with the CS protein binding inhibition assays shown in Fig. 3. In contrast, hyaluronic acid, and chondroitin sulfates A, B, and C added at the same concentrations produced limited or probably no inhibition of hepatocyte infection since adding fivefold higher concentrations of chondroitin sulfate A or C had little effect. Thus, these data indicate that certain sulfated glycoconjugate structures clearly effectively inhibit sporozoite invasion of hepatocytes while others give little or no inhibition.

The time course for the inhibition of hepatocyte infection by sulfated glycoconjugates was also examined (Table II). Addition of dextran sulfate 5,000 to the hepatocyte culture media for 3 h, rinsing the inhibitor away, and then adding sporozoites produced little or no decrease in the number of cells infected. Similarly, combining sporozoites and hepatocytes for 3 h, rinsing unbound sporozoites away, and then adding inhibitor to the culture media for 21 h had no effects on the number of infected cells. A major reduction in hepatocyte infection was only observed when inhibitor was added at the same time as sporozoites. Therefore, these data suggest that sulfated glycoconjugates inhibit sporozoite infec-

Table III. Inhibition of Sporozoite Infectivity in Mice by Sulfated Glycoconjugates*

Inhibitor	Infected‡	Trials	Mice (total)§
	%		
Dextran sulfate 500,000	44 ± 10	3	18
Dextran sulfate 5,000	100 ± 0	2	12
Fucoidan	17 ± 14	4	24
Heparin	81 ± 25	6	34
Chondroitin sulfate C	83 ± 29	3	18
Hyaluronic acid	100 ± 0	3	18
PBS	94 ± 14	6	34

* Sporozoites were combined with inhibitor (1 mg/ml) for 15 min, and 0.1 ml was injected i.v. per mouse. Blood smears were examined for 21 d, or until the animal became parasitemic.
‡ Mean infected ± σ_{n-1} for the trials was calculated by averaging the number of mice infected per mice injected in each trial.
§ Between 5 and 7 mice were injected with the test inhibitors in each trial.

Discussion

The mechanisms by which *Plasmodium* sp. sporozoites locate, bind to, and invade hepatocytes are not known but undoubtedly involve a number of processes, which in molecular terms must be highly complex. CS protein is thought to play a critical role in mediating the invasion process since (a) CS proteins densely coat sporozoites; (b) the cell surface expression of CS proteins is thought to correlate with sporozoite infectivity (for reviews see reference 13); and (c) antibodies against CS proteins can inhibit invasion (1, and references therein). Several recent reports have pursued a determination of how CS protein may function on the surface of sporozoites. Robson et al. (20) have noted that CS protein, as well as a malaria blood-stage protein, thrombospondin-related anonymous protein, contain amino acid sequences that are homologous to sequences in thrombospondin and properdin. Holt et al. (7) found that a portion of this homology, based on the sequence Cys-Ser-Val-Thr-Cys-Gly-x-Gly-x-x-x-Arg-x-Arg/Lys, occurs in several proteins which bind specifically to sulfated glycoconjugates. Therefore, it was proposed that this sequence may constitute a sulfated glycoconjugate-binding domain. This possibility was supported by research indicating that several otherwise-unrelated proteins which contain the homology sequence have remarkably similar sulfated glycoconjugate-binding characteristics (8).

In this report we have investigated the hypothesis that CS protein binds selectively to sulfated glycoconjugates and have tested the effects of these compounds on sporozoite infectivity. We have shown that a recombinant *P. yoelii* CS protein binds avidly to heparin-, fucoidan-, and dextran sulfate-Sepharose, but binds poorly to chondroitin sulfate A- or C-Sepharose. The CS protein binds less extensively to a heparan sulfate biosynthesis-deficient mutant compared with wild-type cells, and binding to hepatocytes is inhibited by fucoidan, pentosan polysulfate, and heparin, less so by dextran sulfate 500,000 and the chondroitin sulfates, and not at all by hyaluronic acid. Sporozoite invasion of hepatocytes is well inhibited by fucoidan, heparin, and the dextran sulfates, and little or not at all by the chondroitin sulfates and hyaluronic acid. Furthermore, we have determined that sulfated glycoconjugates inhibit an event of sporozoite invasion into hepatocytes which occurs within 3 h of combining the cells. Finally, we show that sporozoite infectivity in mice is also substantially inhibited by fucoidan and high molecular weight dextran sulfate.

Overall, our observations indicate that CS protein binds to selected sulfated glycoconjugates and that these same compounds have a major effect on in vitro *P. berghei* sporozoite infectivity. A subset of this same group of compounds clearly inhibits in vivo infectivity as well. The molecular basis for these different observations and their possible similar or dissimilar mechanistic basis with respect to the effects of sulfated glycoconjugates remains to be determined. However, our observations are generally consistent with the possibility that interactions of sporozoites with hepatocyte cell surface sulfated glycoconjugates represent a step in the invasion process. Inhibition of sporozoite infectivity by sulfated glycoconjugates could then result from their competing with target cell sites for sporozoite surface ligands. This possibility is supported by our observed selective binding of *P.*

berghei sporozoites to sulfatide and the greatly reduced binding of CS protein to a CHO cell mutant having reduced production of sulfated glycoconjugates, especially of heparan sulfate, the primary sulfated glycoconjugate of hepatocyte surfaces (11, 22, 23). Published results indicating that invasion by *Plasmodium* sp. sporozoites is associated with binding of sporozoite components to hepatocyte protein components (24) are not necessarily inconsistent with the possible interaction of sporozoite components, even the same components, with target cell sulfated glycoconjugates. For example, the uptake of fibroblast growth factor by target cells is known to be a two-stage process, a required low-affinity interaction with cell surface heparan sulfate followed by high-affinity binding to a specific protein receptor in the cell membrane (for review see reference 9).

In considering that CS proteins may bind to sulfated glycoconjugates via the described homology sequence, it may be noted that the cell adhesive properties of selected peptides and protein fragments containing such a sequence have been previously demonstrated in other systems. Prater et al. (16) have shown that peptides which include the homology sequence occurring in thrombospondin are strongly adhesive for melanoma cells, and that cell binding to these peptides can be selectively inhibited by the sulfated glycoconjugates, heparin, fucoidan, and dextran sulfate, the same compounds showing greatest activity in our studies. Hematopoietic cell lines have been observed to adhere specifically to surfaces coated with synthetic peptides based on a portion of the homology sequence occurring in CS protein, including ones as small as Val-Thr-Cys-Gly (18). What on the surfaces of the hematopoietic cells is bound by, or binds to, these peptides has not been determined. Selective binding of sulfated glycoconjugates to proteins not having such a region of sequence homology or by binding to sequentially unrelated regions, as is seen for the heparin binding site initially described on thrombospondin (16), has also been observed in several studies, indicating that protein-sulfated glycoconjugate interactions may involve other types of mechanisms as well.

A consideration in the hypothesis that CS proteins and sporozoites interact with sulfated glycoconjugates is how specificity might enter into such an interaction. In this report, we have shown that CS protein, like several other proteins described in the past (5, 7, 8, 19, and references therein), binds strongly to the sulfated glycoconjugates, heparin and fucoidan, but only weakly to chondroitin sulfate A or C. Based on work in other systems, it is possible that CS proteins bind to only specific structures within a class of sulfated glycoconjugates and each class can show enormous structural diversity. For example, heparan sulfates can theoretically occur as 10^{36} different structures (3). However, antithrombin III only binds to specific pentasaccharides out of the enormous diversity of possible heparin structures (14). Furthermore, sulfated glycoconjugates are known to be both species and tissue specific. For example, heparan sulfates isolated from rat liver differ significantly in size and fine structure (sulfation, acetylation, and branch positions) from those in rat kidney or human liver (3, 10, 11, 23).

In considering the mechanisms by which selected sulfated glycoconjugates may inhibit sporozoite infectivity it should also be noted that sporozoites are now known to contain at

least one other surface protein besides CS protein with a similar region of sequence homology, the sporozoite surface protein 2 of *P. yoelii* sporozoites (6).

It is of interest that the inoculation of *P. berghei* sporozoites with low levels of dextran sulfate 500,000 or fucoidan can prevent infection of mice and that this is consistent with the effects of these same compounds on sporozoite infectivity in in vitro assays. Although it is unclear why heparin and dextran sulfate 5,000, which has a similar structure and charge density as dextran sulfate 500,000, inhibit in vitro but not in vivo infectivity, it may be relevant that both are of much lower molecular weight than the inhibitory compounds. A requirement of high molecular weight for a sulfated glycoconjugate to be inhibitory in vivo could result for various reasons, such as the relative absolute levels of high and lower molecular weight compounds required for inhibition (not determined in this study) and their relative rates of dilution in or loss from the blood stream. Besides, in vivo inhibition may involve mechanisms in addition to those operating in in vitro inhibition, such as the blocking of processes associated with the movement of sporozoites through the blood stream or their uptake by endothelial or Kupffer cells in the liver before the invasion of hepatocytes.

A further understanding at the molecular level of the mechanisms leading to inhibition of sporozoite infectivity by sulfated glycoconjugates should provide information useful for understanding the molecular and cellular basis for the sporozoite invasion process. This will hopefully suggest additional approaches for limiting the initiation of plasmodial infections by targeting specific molecular processes for the development of novel vaccines and drugs.

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