**A Toxoplasma Lectin-like Activity Specific for Sulfated Polysaccharides Is Involved in Host Cell Infection**

(Received for publication, October 26, 1998)

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**Toxoplasma gondii** is one of the most widespread parasites of humans and animals. The parasite has a remarkable ability to invade a broad range of cells within its mammalian hosts by mechanisms that are poorly understood at the molecular level. This broad host cell specificity suggests that adhesion should involve the recognition of ubiquitous surface-exposed host molecules or, alternatively, the presence of various parasite attachment molecules able to recognize different host cell receptors. We have discovered a sugar-binding activity (lectin) in tachyzoites of *T. gondii* that plays a role in erythrocyte agglutination and infection of human fibroblasts and epithelial cells. The ability to agglutinate erythrocytes can be reversed by a variety of soluble glycoconjugates, of which heparin, fucoidan, and dextran sulfate were the most effective. Interestingly, infectivity of tachyzoites for human foreskin fibroblasts, cells that are commonly used to grow *T. gondii* in vitro, was increased by low concentrations of the sulfated glycoconjugates that inhibited hemagglutination activity (i.e. dextran sulfate and fucoidan) whereas high concentrations inhibited parasite infection. Furthermore, inhibition of glycosaminoglycan biosynthesis and sulfation on the host cells reduced *Toxoplasma* infectivity. Finally, *Toxoplasma* tachyzoites showed a reduced ability to infect epithelial cell mutants deficient in the biosynthesis of surface proteoglycans. The probable identity of the hemagglutinin(s) was investigated by 1) direct binding of red blood cells to filter blots of *Toxoplasma* proteins separated by polyacrylamide gel electrophoresis, and 2) binding of metabolically labeled parasite proteins to fixed mammalian cells. Three parasite bands were thus identified as candidate adhesins. These results suggest that attachment of *T. gondii* to its target cell is mediated by parasite lectins and that sulfated sugars on the surface of host cells may function as a key parasite receptor.

The protozoan parasite *Toxoplasma gondii* infects about ⅓ of the adult population of the world (1). Although toxoplasmosis is generally asymptomatic in healthy adults, the infection may cause severe sequelae in neonates and life-threatening lesions in AIDS patients (2–4). Furthermore, toxoplasmosis is also of considerable importance in domestic animals, given its association with abortion in sheep and swine and as a major source of infection for humans (5).

The *T. gondii* life cycle includes three major, distinct stages: sporozoites, which are the product of the sexual cycle, and the asexually dividing tachyzoites and bradyzoites. The sporozoite and the bradyzoite reside within distinct cyst structures that are relatively long-lived and infectious if ingested. On the other hand, the rapidly growing tachyzoite is responsible for spreading the infection from cell to cell and probably for most of the disease symptoms. Tachyzoites multiply inside a parasitophorous vacuole that does not fuse with host cell lysosomes. Rapid multiplication of the intracellular parasite is followed by rupture of the host cell and infection of contiguous cells. At some point during infection, there is a shift from multiplication of tachyzoites to the formation of tissue cysts filled with bradyzoites (5).

*Toxoplasma* tachyzoites have the ability to interact with and invade a diverse array of cells, such as fibroblasts, epithelial cells, endothelial cells, macrophages, and cells of the central nervous system. Ultrastructural studies indicate that invasion is a very rapid (15–40 s) and complex event in which specialized organelles located in the anterior pole of the tachyzoite seem to be involved (6–8). The process of entry is initiated by the parasite contacting the host cell with its anterior region; this is followed by protrusion of the conoid, modification of the host cell membrane, and secretion of the microneme and rhoptry contents. As the parasite enters, host cell pseudopods extend along the parasite, so that the tachyzoite becomes enclosed in an intracellular vacuole separated from the host cytosol by a unit membrane (6–8).

Attachment is a prerequisite for microbial colonization and invasion of host cells and is usually mediated by microbial surface proteins called adhesins that bind to protein or carbohydrate epitopes present on the host cell surfaces. Several lines of evidence indicate that *Toxoplasma* tachyzoites interact with host cells by specific receptor-ligand types of interactions (9–11). For instance, invasion of host cells by tachyzoites can be inhibited by treating the parasite with rabbit polyclonal and mouse monoclonal antibodies against the *Toxoplasma* major surface protein, SAG1 (9). However, the fact that a SAG1-deficient mutant is still able to infect host cells (9) suggests that additional parasite molecules may be available to allow host cell infection. That components of the extracellular matrix may be important participants in this process has also been shown in experiments demonstrating that the glycoprotein laminin was able to enhance tachyzoite attachment to a macrophage-like cell line in vitro (10). Subsequently, it was reported that host cell laminin bound to the parasite surface mediates binding of the tachyzoite to the laminin receptor α6β1 on eukaryotic cells (11).

The knowledge that *T. gondii* possesses the ability to attach...
and to replicate within almost any type of nucleated cell prompted us to look for clues that may explain this promiscuous and efficient parasite conduct. One possible interpretation for this seemingly indiscriminate behavior may be the presence of multiple adhesion molecules on the Toxoplasma surface that interact with cells of dissimilar background and surface composition. However, it is also possible that a single Toxoplasma adhesin may be able to recognize a host cell surface receptor common to most vertebrate cells. Because carbohydrates are found modifying most cell surface proteins and have the greatest potential for structural variety, and because there is good evidence to believe that they act as carriers of biological information, our studies were designed to identify a potential T. gondii sugar-binding protein that might be involved in the process of host cell recognition and attachment. We have discovered a Toxoplasma surface lectin with specificity for sulfated polysaccharides that appears to be important in the process of attachment to the host cell.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents—**Animal cell mutants defective in glycosaminoglycan biosynthesis were derived from Chinese hamster ovary (CHO) K1 cells and were kindly provided by Jeffrey D. Eako (University of California, San Diego) (12–14). These mutants, pgsA-745, pgsB-761, pgsD-677, pgsE-606, and pgsB-650, are defective in xylosyltransferase, galactosyltransferase I, heparan sulfate synthesis, heparan sulfate N-sulfotransferase, and galactosyltransferase I, respectively. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO₂ in Ham’s F-12 medium supplemented with 10% fetal bovine serum, containing 2 mM glutamine, 100 ng/ml penicillin and 100 units/ml streptomycin. Dextran sulfate (MW, 8,000, 50,000, and 500,000), fucoidan; chondroitin sulfate A, B, and C; heparan sulfate; hyaluronan; dextran (MW, 500,000); p-nitrophényl-β-D-xylan osidase; p-nitrophényl-α-D-xylan osidase; and glucuronidase, as well as all monosaccharides and glycoproteins, were purchased from Sigma. FITC-conjugated heparin was obtained from Molecular Probes. Monoclonal antibodies against heparin (MAB570 and MAB2040), heparan sulfate proteoglycan core protein (MAB458), and chondroitin-4-sulfate (MAB2030) were obtained from Chemicon International Inc. Monoclonal antibody against chondroitin-6-sulfate (MCA277) was obtained from Serotec. All other reagents were analytical grade or the best available commercial grade.

**Parasite Cultures—**Toxoplasma strains RH and PDS were maintained by serial passage every 2–3 days in confluent monolayers of primary HFF cells as described previously (15). HFF cells were grown at 37 °C in Dulbecco’s modified essential medium supplemented with 5% Nu-serum, containing 2 mM glutamine, 100 ng/ml streptomycin. Extracellular tachyzoites were isolated by filtration, followed by centrifugation at 500 g for 5 min at 4 °C. Alternatively, parasites were obtained by scraping the infected monolayer into a suspension immediately after harvesting by addition of 3% buffered-formalin as above, and detergent solubilization were used in hemagglutination assays with glutaraldehyde-fixed erythrocytes. Thus, a sample (25 μl) of Toxoplasma organisms or lysate was serially diluted (2-fold) using PBS, 0.5% BSA as diluent. The assay was initiated by the addition of 25 μl of a 2% suspension of fresh or glutaraldehyde-fixed rabbit erythrocytes. After 1 h at room temperature, each well was examined for agglutination. Lectin titers were defined as the reciprocal of the highest dilution of parasites capable of causing detectable agglutination of erythrocytes, as determined by visual inspection. In all experiments, serial dilutions of potential inhibitors (25 μl) were mixed with an equal volume of a concentration of parasites 4-fold higher than the minimum concentration required to give complete agglutination, for 30 min at room temperature. At the end of this time, 25 μl of a 2% suspension of erythrocytes was added, and the minimum concentration causing inhibition recorded after 60 min at room temperature.

**T. gondii Infection Assay—**HFF cells were harvested by trypsinization, resuspended in DMEM supplemented with 5% Nu-Serum, plated at ~1 × 10⁵ cells/well in 24-well plates (Falcon), and incubated overnight at 37 °C. The next day, cell monolayers were washed twice with serum-free DMEM and incubated at 37 °C in 0.25 ml of 0.5% BSA, DMEM until needed. Tachyzoites were harvested, resuspended at ~4 × 10⁷ organisms/ml and 0.25 ml was added to duplicate monolayers and incubated at 37 °C. One hour after infection, cultures were washed three times with serum-free DMEM and incubated in DMEM supplemented with 5% Nu-serum. Toxoplasma infection was determined by the selective incorporation of [³H]uracil by replicating parasites as described previously (17). For inhibition experiments, tachyzoites were preincubated with various concentrations of inhibitor for 30 min at 37 °C in 0.5% BSA, DMEM. At the end of the incubation time, the mixture was added to cell monolayers and incubated at 37 °C for 1 h. Then, cell monolayers were washed three times with serum-free DMEM to remove unattached organisms and further incubated in DMEM supplemented with 5% Nu-Serum overnight at 37 °C, and infection rate was determined as described above. To assess the role of host cell surface proteoglycans, T. gondii infectivity was determined using confluent monolayers of animal cell mutants pgsA-745, pgsB-761, pgsD-677, pgsE-606, and pgsB-650, which are defective in glycosaminoglycan biosynthesis, and wild type K1 cells as described (18).

**T. gondii Attachment Assay—**T. gondii attachment was determined using confluent monolayers of animal cell mutants pgsA-745 and pgsB-761, which are defective in glycosaminoglycan biosynthesis, and wild type K1 cells grown in 24-well tissue culture plates (Falcon) by addition of 1% glutaraldehyde (19). Toxoplasma tachyzoites were harvested, preincubated in methionine-free DMEM for 30 min at 37 °C, and metabolically labeled by addition of 250 μCi/ml of [³⁵S]methionine for 1 h at 37 °C. At the end of the incubation time, organisms were washed three-times in DMEM and resuspended at ~2 × 10⁷ cells/ml in 0.5% BSA, DMEM. To assay binding, 0.5 ml was added to duplicate monolayers of confluent monolayers of animal cell mutants pgsA-745, pgsB-761, pgsD-677, pgsE-606, and pgsB-650, which are defective in glycosaminoglycan biosynthesis, and wild type K1 cells as described (18).

**Binding of FITC-conjugated Heparin—**Freshly harvested organisms were washed in PBS, incubated with increasing concentrations of FITC-heparin for 1 h at 4 °C, and washed three times with ice-cold PBS, and organism-associated fluorescence was determined using an Olympus BX60 fluorescence microscope. Alternatively, live organisms were fixed in suspension immediately after harvesting by addition of 3% buffered-formalin for 10 min at 4 °C, incubated in 0.1 μl glycine, PBS, pH 7.2, washed in PBS, pH 7.2, and used intact or after permeabilization with 0.25% Triton X-100. In another set of experiments, parasite aliquots were dried on glass slides, fixed with buffered-formalin as above, and used for binding assays. Binding specificity was evaluated by using 0.5 μg/ml of FITC-labeled heparin and competition with varying concentrations of unlabeled potential inhibitors.

**Indirect Fluorescent Antibody Staining—**Cellular localization of glycosaminoglycans was examined using normal antibodies specific for galactosaminoglycan core protein (MAB458, IgG1), chondroitin-4-sulfate (MAB2030, IgG1), and chondroitin-6-sulfate (MCA277, IgM). For evaluation of extracellular organisms, tachyzoites were dried on glass slides, fixed with buffered-formalin for 10 min at room temperature in 3% buffered-formalin, washed in 0.1 μl glycine, PBS, pH 7.2, and incubated in the appropriate concentration of antibodies in 1% BSA, PBS, pH 7.2 for 30 min at room temperature.
parasites, confluent monolayers of HFF were grown in 8-well chamber slides overnight, infected by addition of tachyzoites, and incubated for 48 h. Infected cell monolayers were rinsed with PBS, pH 7.2, fixed in 3% buffered-formalin as above, permeabilized by preincubation in 0.25% Triton X-100, PBS, pH 7.2, and probed using ~5 μg/ml of specific monoclonal antibodies, followed by a goat anti-mouse IgG or IgM conjugated to fluorescent isothiocyanate (Sigma) diluted 1:50 in PBS, 1% BSA. Negative control included normal mouse serum (1:100 dilution) or incubation with a goat anti-mouse IgG or IgM conjugated to fluorescein isothiocyanate.

Inhibition of Proteoglycan Biosynthesis and Sulfitation by HFF Cells—To inhibit the synthesis of host cell proteoglycans, live HFF cell monolayers grown in 24-well plates were preincubated with 2 mM p-nitrophenyl-β-D-xylopyranoside DMEM in 2% diazylated fetal calf serum overnight at 37 °C and then used for infection assays. As a control, monolayers were incubated with 2 mM p-nitrophenyl-α-D-xylopyranoside, which does not inhibit proteoglycan biosynthesis. To inhibit sulfation of glycosaminoglycans, HFF cells were cultured overnight in 24-well plates in DMEM supplemented with 2% diazylated fetal bovine serum and NaClO₃, a reversible inhibitor of adenosine 3’-phosphoadenosine 5’-phosphosulfate and proteoglycan sulfation or NaCl as a control to a final concentration of 60 mM, as described (20). Monolayers were washed once with cold DMEM and analyzed as described above for T. gondii infection assay.

Identification of Hemagglutinating Factors—Toxoplasma tachyzoites were harvested as explained above, washed once in serum-free DMEM, resuspended in methionine-free DMEM supplemented with 2% diazylated bovine serum and incubated for 30 min at 37 °C. Then, parasite proteins were metabolically labeled by addition of 250 μCi/ml of [35S]methionine for 1 h at 37 °C. At the end of the incubation time, organisms were washed three times in PBS, pH 7.2, resuspended at ~2 × 10⁶ cells/ml, and sonicated at 4 °C (three cycles of 30 s at 25 W). Cell debris was pelleted by centrifugation at 500 × g for 10 min at 4 °C. Subcellular fractions were obtained by centrifuging the sonicated supernatant at 100,000 × g for 1 h at 4 °C to give a vesicular membrane pellet and soluble cytoplasmic components. The membrane pellet was then solubilized in 1% Triton X-100 for 1 h at 4 °C in the presence of antiprotease mixture (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of aprotinin, leupeptin, soybean trypsin inhibitor, and pepstatin) and centrifuged as above to obtain a cytoskeleton pellet and soluble membrane fraction. Finally, the cytoskeleton pellet was solubilized in 1% SDS for 1 h at 20 °C and centrifuged as above to obtain a soluble cytoskeleton fraction.

For identification of hemagglutinating factors, a 100-μl aliquot of the different labeled-parasite fractions was mixed with 100 μl of a 25% (v/v) suspension of 1% glutaraldehyde-fixed rabbit red blood cells or HFF cells and 100 μl 1% BSA in PBS and incubated for 1 h at room temperature. The cytoskeleton fraction in addition received 700 μl of 2% Triton X-100, 100,000 × g for 10 min and filtration through 0.45 μm filter showed strong hemagglutinating activity in the cytoskeleton fraction. The ability to agglutinate erythrocytes has been widely used to detect and characterize microbial factors that mediate host cell attachment (22, 23). The specificity of this interaction can be assessed by exploiting the large variability of glycoproteins and glycolipids on the surface of erythrocytes of various animal species (23, 24). The method is semiquantitative because it measures hemagglutination titers by doubling dilutions, but is very reproducible, easy to perform, and highly flexible (22, 23). In preliminary experiments, hemagglutination assays were performed at room temperature using purified, live T. gondii as the source of presumptive lectin; the results showed that the parasites were very effective in agglutinating fixed rabbit red blood cells, with a minimum of 10⁴ parasites required to produce visible hemagglutination (data not shown).

The fact that significant activity was observed during incubation at 4 °C (which should prevent parasite secretory/excretory activity) suggested that the hemagglutinating activity was associated with the parasite surface. However, a hemagglutinating activity was also detected in a soluble, secreted material, because parasite-free supernatants, obtained by incubating tachyzoites in serum-free DMEM for 1 h at 37 °C, followed by centrifugation at 500 × g for 10 min and filtration through a 0.45 μm filter showed strong hemagglutinating activity in the conditioned media. Subsequent experiments were performed using glutaraldehyde-fixed erythrocytes and lysates of T. gondii in an attempt to extend the range of conditions to be tested. Subcellular fractionation (Fig. 1) showed that the strongest hemagglutinating activity was located in the cytoskeleton fraction with a lesser amount in the soluble membrane material, whereas the cytosol had no detectable activity. This result implies that part of the hemagglutinin activity is localized in the membrane fraction. Interestingly, detergent lysates of the cytoskeleton fraction were more active than total lysates, despite the presence of a relatively high concentration (~0.4%) of the cationic detergent SDS. This suggests that the active site of the hemagglutinin is highly stable, that by denaturing the molecule a second binding domain is exposed, and/or that some of the hemagglutinin is contained within detergent-soluble constituents. No differences in hemagglutinating activity were observed when the assay was performed at 20, 37, or 40 °C, but a decline of 50% in activity was observed after incubation at

**RESULTS**

Identification of a T. gondii Sugar-binding Protein—The ability to agglutinate erythrocytes has been widely used to detect and characterize microbial factors that mediate host cell attachment (22, 23). The specificity of this interaction can be assessed by exploiting the large variability of glycoproteins and glycolipids on the surface of erythrocytes of various animal species (23, 24). The method is semiquantitative because it measures hemagglutination titers by doubling dilutions, but is very reproducible, easy to perform, and highly flexible (22, 23). In preliminary experiments, hemagglutination assays were performed at room temperature using purified, live T. gondii as the source of presumptive lectin; the results showed that the parasites were very effective in agglutinating fixed rabbit red blood cells, with a minimum of 10⁴ parasites required to produce visible hemagglutination (data not shown).

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Sugar specificity was determined by a hemagglutination inhibition assay. The minimal inhibitory concentration is defined as the minimal concentration of a sugar required to inhibit 4 hemagglutination units of lectin activity.

| Inhibitors | Minimal inhibitory concentration mg/ml |
|------------|--------------------------------------|
| Fucoidan   | 0.0006                               |
| Dextran sulfate | 0.0006                               |
| Heparin    | 0.0006                               |
| Asialofetuin | 0.156                               |
| Heparan sulfate | >1.0                                |
| Hyaluronan | 2.5                                  |
| Dextran    | >5.0                                 |
| Chondroitin sulfate A | >5.0                                |
| EDTA       | 200 mm                               |
| EGTA       | 200 mm                               |

*Saccharides that were ineffective in inhibiting hemagglutination included (highest concentration tested in mg/ml) mucin I-S (5), NANA (5), xylose (50), galactose (50), GaINAc (50), GlcNAc (50), lactose (50), mucin type II (5), chondroitin sulfate B and C (5), keratan sulfate (5), collagen type VII (5), thyroglobulin (5), methyl-α-mannoside (5), mannan (5), mannose (50), N-fucose (50), ovalbumin (5), mannosamine (50), glucose (50), glucosamine (50), (GlcNAc)2 (1), (GlcNAc)3 (1), and rhamnose (50).

56 °C for 5 min, and heating at 100 °C almost completely abolished hemagglutination (~90% decrease).

If the hemagglutinin described above is a sugar-binding protein, it would be expected that red cell agglutination should be inhibited by soluble glycoconjugates. A total of 33 simple (mono- and disaccharides) and complex (oligosaccharides, glycoproteins, and proteoglycans) saccharides were tested for their ability to inhibit hemagglutination. The relative potency of the inhibitors is shown in Table I. Of the 33 examined, 28 showed no inhibition, even at the highest concentrations tested (5–50 mg/ml). The remaining 5 showed various degrees of inhibition, with minimal inhibitory concentrations ranging from 0.0006 to 0.156 mg/ml. The most striking feature of this analysis was that sulfated sugars, including heparin, fucoidan, and dextran sulfate, were the most effective inhibitors of hemagglutination. Uncharged and carboxylated polymers, as well as most glycoproteins, were ineffective or comparatively poor inhibitors. The fact that asialofetuin was as potent an inhibitor as fetuin suggests that sialic acid is not involved in the attachment to the host cell.

The inability of dextran to inhibit hemagglutination suggests that sulfation of the polysaccharide is critical for agglutination to occur. Evidence for a specific steric requirement is based on the fact that inhibition of hemagglutination was not related to the charge density on the polysaccharides. Fucoidan, for example, inhibited agglutination at 600 ng/ml and has a negative charge density of 0.3/mol, whereas chondroitin sulfate and dermatan sulfate with a negative charge density of 1.0/mol, did not inhibit agglutination at >5 mg/ml (Table II). In fact, the inability of heparan sulfate, closely related to heparin, to inhibit hemagglutination even at 1 mg/ml, suggests that the spatial orientation of sulfated groups and the composition of the polymer chain strongly affect the affinity of binding. Several other glycoconjugates, including bovine submaxillary mucin, hog gastric mucin, thyroglobulin, and ovalbumin, did not inhibit hemagglutination. In addition, neither EDTA nor EGTA at 200 mM inhibited hemagglutination, suggesting that exogenous divalent cations are not important for lectin activity to occur or that Ca2+ is tightly bound and is not released in the presence of the chelating agents. All the strains tested so far, including the RH and ME49 strains and a SAG1-deficient mutant of ME49, have shown hemagglutinating activity and similar sugar specificity, indicating that the activity is not a peculiarity of one strain (data not shown).

Infection of HFF Cells by T. gondii Is Stimulated by Glycoconjugates—Incubation of T. gondii tachyzoites with HFF monolayers leads to rapid invasion by the parasite, a process that can be readily evaluated by light microscopy and quantitated by staining the host cells and counting the number of infected cells and intracellular organisms. Investigators in the field have also exploited the ability of the parasite, but not the host cell, to incorporate [3H]uracil, to more rapidly quantitate the number of intracellular T. gondii (17). However, because this assay does not discriminate between failure to invade and failure to grow once inside the cell, specific methods that do make this distinction must also be used (see below).

To test the hypothesis that T. gondii binding to host cells is mediated by a sugar-protein type of interaction and given our preliminary findings using erythrocytes as target cells, we attempted to block the infectivity of Toxoplasma (defined as invasion plus growth) to HFF cells with soluble glycoconjugates of known structure. Using a limited number of compounds, we found that the infectivity was not inhibited by any of the glycoconjugates tested except at exceptionally high concentrations (see below). On the contrary, we observed that addition of fucoidan and dextran sulfate increased the infectivity of the parasite by 2.8- and 1.8-fold, respectively, when tested at 100 μg/ml (Fig. 2), and that the effect was dose-dependent with maximal stimulation in the 10–100 μg/ml concentration range, but, at least for fucoidan, the enhancement was lost at 500 μg/ml. Increase in infection was specific, as other glycoconjugates, such as heparin, chondroitin sulfate, and keratan sulfate, were not effective in stimulating Toxoplasma infectivity (Fig. 2 and data not shown). Interestingly, the glycoconjugates that showed the infectivity-enhancing activity were also two of the most effective inhibitors of the hemagglutinating activity. The results in Fig. 2 can be interpreted on the basis of a lectin activity present on the surface of T. gondii and HFF cells that shares the same carbohydrate specificity. Therefore, at low concentrations, simultaneous binding of the same ligand will promote cell-cell interaction and increase infectivity. Whereas additional increase in the dose of heparin and dextran sulfate (1.25–5 mg/ml) produced a marked decrease (80%) in Toxoplasma infectivity (data not shown), interpretation of the results at these high concentrations is very tentative because of the possibility of nonspecific effects on the cultures. Interestingly, even the highest concentrations of fucoidan (5 mg/ml)
failed to inhibit *Toxoplasma* infectivity suggesting a large number of surface receptors or coaggregation of the polysaccharide on the tachyzoite cell surface, as has been suggested for other organisms (25).

**Proteoglycan Biosynthesis and Sulfation Are Required for Maximal Infectivity of Mammalian Cells by *T. gondii*—**The observation that only sulfated polysaccharides inhibited cell agglutination and modified infectivity suggested that sulfation of the polysaccharide was important for parasite recognition. We first tested the ability of tachyzoites to infect HFF cells grown in the presence of *p*-nitrophenyl-β-D-xylopyranoside, a soluble acceptor for glycosaminoglycan polymerization that competes with the endogenous proteoglycan core protein acceptor, resulting in diminished cell surface proteoglycans (26). Under these conditions we observed a reduction of 60% in the ability of *Toxoplasma* to invade and/or grow in HFF monolayers as compared with control cells grown in normal media (Fig. 3A). Controls treated with the inactive α-form of the drug showed little, if any, reduction in infectivity (Fig. 3A).

To further evaluate the involvement of glycosaminoglycans in *Toxoplasma* infection, we next used sodium chlorate to interfere with sulfation reactions (27, 28). Incubation of cell monolayers in the presence of chlorate reduced the infectivity by 59 or 68% as compared with cells grown in media containing NaCl at similar ionic strength or normal DMEM, respectively (Fig. 3B). Removal of chlorate from the culture medium of cells just prior to infection resulted in essentially full restoration of host cell susceptibility (data not shown).

**Reduced *T. gondii* Infectivity for epithelial cell mutants deficient in proteoglycan biosynthesis—**Because selectivity is not easily achieved with any of the available pharmacological inhibitors, we next examined the interaction of *Toxoplasma* with cell lines carrying genetic defects in glycosaminoglycan assembly. Such animal cell mutants have been derived from CHO cells by Esko and co-workers (12–14) and offer the sought selectivity. Thus, on the basis that *Toxoplasma* expresses a lectin activity that is best inhibited by the proteoglycan heparin and sulfated polysaccharides (i.e. fucoidan and dextran sulfate), we used five CHO cell mutants (pgsA-745, pgsB-761, pgsD-677, pgsE-606, and pgsB-650) defective in proteoglycan biosynthesis to test for the role of those molecules in mediating host cell infection by this organism. All the mutants lack or have reduced amounts of heparan sulfate and other heparin-like sequences present in wild type cells. The results showed that all five proteoglycan-deficient mutants supported *T. gondii* infection to a much lesser extent than wild type cells (Fig. 4). Four independent experiments performed in triplicate have consistently showed a reduction of about 63–80% compared with parental cells. Mutants pgsA-745, pgsB-761, and pgsB-650 make 5, 8, and 32%, respectively, of the amount of proteoglycan synthesized by wild type cells (12). The fact that mutant pgsD-677 does not synthesize any heparan sulfate but contains about three times more chondroitin sulfate than the wild type cells suggests that heparan sulfate, but not chondroitin sulfate, mediates infection of *Toxoplasma*. Because mutant pgsE-606 produces heparan sulfate that is 2–3 fold undersulfated but normal amounts of fully sulfated chondroitin sulfate (13), we conclude that sulfation of the heparan chain is important for optimal infection. These findings support the hypothesis that proteoglycan-mediated infection plays an important role during *Toxoplasma* infection.

**Attachment of Toxoplasma to cells deficient in proteoglycan synthesis is reduced—**Two different approaches were taken in order to evaluate whether the differences observed in *Toxoplasma* infectivity of epithelial cell mutants deficient in proteoglycan biosynthesis were related to early events in the infection

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**FIG. 2.** Stimulation of *T. gondii* infectivity by soluble proteoglycans. Tachyzoites were harvested, mixed with proteoglycans at the indicated concentrations, and allowed to infect a monolayer of HFF for 1 h at 37 °C. Then, monolayers were washed, and the number of intracellular organisms was determined by the selective incorporation of [*3H*]uracil over 16 h. Values shown are the mean ± S.E.

**FIG. 3.** Infection of HFF cells treated with inhibitors of proteoglycan synthesis and sulfation. A, HFF cells were grown in the presence of 2 mM *p*-nitrophenyl-β-D-xylopyranoside or *p*-nitrophenyl-α-D-xylopyranoside in 2% dialyzed fetal calf serum and DMEM overnight at 37 °C and used in *Toxoplasma* infection assays. B, HFF cells were treated overnight with 60 mM NaClO₃ or NaCl in 2% dialyzed fetal calf serum and DMEM and used in *Toxoplasma* infection assays as described. Each value represents the mean ± S.E.
process (i.e. attachment/invasion) or to altered intracellular growth. First, metabolically labeled tachyzoites were used to infect live, confluent cells and, after extensive washing and only a short (1 h) incubation at 37 °C, the monolayer-associated radioactivity was then measured. A significant reduction (61–64%) was observed in the ability of Toxoplasma to attach/invas mutant pgsA-745 cells as compared with wild type K1 cells at all the doses evaluated (Fig. 5A), supporting the idea that the differences observed occur early in the infection process. Second, glutaraldehyde-fixed monolayers (i.e. cross-linked cell surface membrane) were evaluated for their ability to support attachment of metabolically labeled parasites. Organisms were gently centrifuged on top of the monolayers to increase the efficiency of binding and the radioactivity associated to the cells was quantitated after 5 and 15 min of incubation at 37 °C. Binding of tachyzoites to mutant pgsA-745 cells was reduced by about 60% in both cases as compared with wild type cells (Fig. 5B), further supporting the idea that diminished attachment accounts for the differences in infectivity.

Fluoresceinated Heparin Binds to Toxoplasma Tachyzoites—Direct binding of heparin to Toxoplasma was tested using FITC-labeled heparin. In preliminary experiments, we found that live, fresh organisms were unable to bind detectable amounts of FITC-heparin at 4 °C (Fig. 6A). Note the presence of a few dead parasites in this preparation that do stain, consistent with the results using permeabilized organisms to be discussed below. After formalin fixation of live organisms and permeabilization with 0.2% Triton X-100, discrete intracellular binding of fluoresceinated heparin was observed within elongated organelles in the anterior region (Fig. 6B). Interestingly, parasites dried on glass slides and fixed with buffered-formalin or methanol showed an intense, peripheral fluorescence (Fig. 6C). This suggests that stimulation from attachment results in lectin activity appearing on the surface of the parasites. In this context, when freshly harvested, live tachyzoites were added to tissue culture-treated chamber slides for 15 min at 37 °C, washed, and fixed with formalin, strong binding of FITC-heparin to the entire parasite periphery was observed, supporting the possibility of induced release once the organism interacts with a substratum (data not shown) (29).

To determine whether there was a single promiscuous glycosaminoglycan receptor or different receptors for heparin, dextran sulfate, and fucoidan, competition experiments were performed in the presence of a fixed amount of FITC-heparin (0.5 μg/ml) and varying amounts of each putative competitor (Fig. 7). Heparin, dextran sulfate, and fucoidan completely inhibited FITC-heparin binding to fixed tachyzoites at 100 μg/ml. The inhibition was specific, inasmuch as neither dextran nor chondroitin sulfate (Fig. 7) inhibited FITC-heparin binding at 100 μg/ml or even at 1 mg/ml (data not shown). These findings are consistent with a common receptor for heparin, dextran sulfate, and fucoidan and with the possibility that Toxoplasma contains a large pool of intracellular heparin-binding molecules that, once released, interact with the parasite surface.

Anti-glycosaminoglycan Antibodies Bind to Toxoplasma Tachyzoites—Because FITC-heparin failed to bind to the surface of live
Live tachyzoites were fixed by addition of 3% buffered formalin, washed, permeabilized with 0.25% Triton X-100, and evaluated for binding of FITC-heparin (b). Tachyzoites were dried on glass slides, fixed with 3% buffered formalin, and evaluated for binding of FITC-heparin (c) as described under “Experimental Procedures.”

**Fig. 7.** Binding of FITC-heparin to tachyzoites is inhibited by sulfated polysaccharides. For competition experiments, fixed tachyzoites were preincubated in an excess of the putative inhibitors (100 μg/ml) (a, noninhibitor; b, heparin; c, chondroitin sulfate; d, dextran; e, dextran sulfate; f, dextran) followed by addition of 0.5 μg/ml FITC-heparin, washed, and evaluated for binding of fluoresceinated heparin.

The parasite-associated proteoglycan-like molecule is currently unknown, but its presence is in agreement with the hypothesis that proteoglycans may play an active role in Toxoplasma-host cell interaction.

**T. gondii Proteins Bind to Erythrocyte and HFF Cell Surfaces**—In an attempt to identify the putative Toxoplasma lectin(s), we took advantage of the known ability of parasite lysates to agglutinate rabbit erythrocytes. Thus, total tachyzoites proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. Filters were equilibrated in Triton X-100, followed by BSA, PBS, pH 7.2, and incubated with a suspension of erythrocytes at room temperature. After gentle rinsing of the filter membrane with PBS, pH 7.2, we observed binding of erythrocytes to three distinct bands of 45, 65, and 71 kDa (Fig. 10A). Preincubation of the filter with soluble fucoidan (1 mg/ml) prevented binding of the 65- and 71-kDa bands (data not shown) but not the 45-kDa band. When nitrocellulose replicas of Toxoplasma subcellular fractions were tested by the erythrocyte binding assay, the pattern of binding closely mimicked the relative hemagglutinating activity observed in lysates, with prominent bands in the membrane and cytoskeleton fractions and a cytosolic fraction almost devoid of binding activity (Fig. 10B).

To gain further insights on the possible identity of the lectin(s), we tested whether metabolically labeled, solubilized T. gondii proteins were able to bind to glutaraldehyde-fixed erythrocytes. For these experiments, parasites were labeled with [35S]methionine, and subcellular fractions were prepared by sonication as described above and used for ligand binding experiments. In preliminary experiments, incubation of fixed erythrocytes with Toxoplasma lysates showed that a limited number of radioactive proteins bind to the surface of fixed cells (Fig. 11). Particularly, proteins with molecular masses of 26,
45, and 65 kDa were eluted from the cell surface and easily detected by SDS-PAGE and autoradiography. Interestingly, binding of the 26- and 65-kDa proteins can be inhibited by soluble heparin and fucoidan (Fig. 11) and dextran sulfate (data not shown), in accordance with the ability of these compounds to inhibit hemagglutination. In contrast, no inhibition of binding for these two bands was observed when many other polysaccharides were tested, including chondroitin sulfate, dextran (Fig. 11), and dermatan sulfate (data not shown). The 45-kDa protein bound with such a high affinity to the surface of the cell that its binding could not be inhibited by any of the glycoconjugates used at the doses tested. When the glutaraldehyde-fixed erythrocytes were substituted with fixed HFF or epithelial (CHO K1) cells in the adsorption experiment, a similar pattern of bands was observed, with the 26-, 45-, and 65-kDa bands binding with equal relative amounts to all cells (data not shown). These combined data indicate that 26- and 65-kDa proteins are strong candidates for the Toxoplasma glycosaminoglycan-binding molecules. The role of the 45-kDa protein awaits further biochemical and immunological studies.

**DISCUSSION**

Cell surface lectins are increasingly being considered as mediators of cell-cell interaction in a range of biological systems (30–32). In the microbial world, lectins help microorganisms in the process of recognition and attachment to host surfaces, a critical step in successful colonization and, ultimately, production of disease (33). In the case of Apicomplexa parasites, there is evidence that the homing of malaria sporozoites to the liver is based on the recognition of hepatocyte cell surface heparan sulfate (reviewed in ref. 34), whereas infection of the host erythrocytes by *Plasmodium* merozoites appears to be mediated at least in part by sialic acid (35), whereas placental chondroitin sulfate A is a cell surface receptor for infected erythrocytes (36). We thus sought to determine whether a Toxoplasma lectin or family of lectins that recognize different sugar residues might modulate Toxoplasma tropism and infection.

For our initial experiments, we chose the method of mixed agglutination using whole tachyzoites and detergent lysates of the parasite plus rabbit erythrocytes and found an agglutinating activity inhabitable by the sulfated polysaccharides heparin, dextran sulfate, and fucoidan. None of the tested mono- and disaccharides inhibited agglutination, suggesting that a polysaccharide is required to span two or more active sites on the sugar binding domain or may be a reflection of the fact that the binding mechanism involves a large number of weak interactions. An important question is whether inhibition of hemagglutination by heparin, dextran sulfate and fucoidan is related to electrostatic interactions or a specific steric configuration, because this would give an insight into the molecular mechanisms involved in these events. Because the glycosaminoglycans, heparan sulfate, hyaluronic acid, chondroitin sulfate, dermatan sulfate, and keratan sulfate have charge densities that are comparable to or higher than fucoidan, and yet they display no inhibitory activity, the inhibitory mechanism must depend on more than the electrostatic interaction between charged molecules of different polarities.

The structural specificity required for inhibition is also exemplified by the difference in potency of heparin and heparan sulfate. Structurally, heparin and heparan sulfate molecules consist of repeated disaccharide units that are sulfated differently. In general, heparin contains a higher proportion of sulfation and higher 1-iduronic acid:3-glucuronic acid ratio than heparan sulfate, suggesting that although a relatively high sulfation density appears essential, it is possible that the composition of the carbohydrate backbone and the position of sulfated groups also play a crucial role in the interaction of the Toxoplasma lectin and its inhibitors. Furthermore, a range of effects on Toxoplasma-host cell interaction, not directly related to their charge density (Table II), was observed with the different sulfated polysaccharides. Therefore, a unique specificity of binding, probably related to the conformation of the native ligand, dictates the biological effect and suggests that the location of the sulfated residues is as important as the charge density per se.

It would be tempting to propose a model on the basis of some common structural motif. For fucoidan, a sulfated polymer of α-(1→3)-linked l-fucose, it has been estimated that most of the sulfated esters are located on the C-4 position (37). However, in the case of heparin the carbohydrate backbone consists of hexuronic acid (O-glucuronic or L-iduronic) β-(1→4)-linked to D-glucosamine units, with variable location of the sulfated substituents, encompassing: N-sulfation of free amino groups at C-2 of glucosamine, O-sulfation at C-2 of iduronic acid residues, and O-sulfation at C-6 of glucosamine units (38). Dextran sulfate is a α-(1→6)-linked D-glucose polymer that is highly O-sulfated. Therefore, the available information does not justify modeling on the basis of their constituent sugars and glycosidic linkage. It is possible that the role of the polysaccharide backbone may be to determine the specificity of binding by providing a correct spatial orientation of the N-acetylated, N-sulfated, and O-sulfated groups, mimicking the region on the host cell...
recognized by the Toxoplasma lectin. Alternatively, it is possible that those agents with the highest sulfate content may make it more likely that a sulfate group is in a favorable context for binding in a substantial fraction of the molecules.

Competition experiments using FITC-heparin and unlabeled inhibitors (e.g., heparin, dextran sulfate, and fucoidan) argue for the existence of a nonspecific parasite receptor capable of interacting with a range of sulfated polysaccharides able to inhibit hemagglutination and alter parasite infection.

The identity of the host cell molecule(s) recognized by this parasite receptor is not known. Among the prominent sulfated macromolecules found in higher cells are the proteoglycans, a class of glycoproteins that contain a core protein with one or more covalently attached glycosaminoglycan chains. Glycosaminoglycans consist of linear polymers composed of disaccharide repeating units of uronic acid and hexosamine. There are four general classes of glycosaminoglycans: 1) hyaluronic acid, 2) chondroitin/dermatan sulfate, 3) keratan sulfate, and 4) heparan sulfate/heparin (39, 40). As the chains polymerize, they undergo various sulfation and epimerization reactions that, together with variations in the length of the polymer, confer proteoglycans with a tremendous structural heterogeneity. Recent studies support the role of cell surface proteoglycans as adhesion receptors for many pathogenic microorganisms (reviewed in Ref. 41), including viruses, protozoan parasites, and bacterial pathogens (19, 25, 42–44). These interactions fall into two classes: (a) Trypanosoma cruzi (19), Leishmania donovani (43), Borrelia burgdorferi (20, 44), and some herpesvirus (42) bind directly to mammalian cell surface proteoglycans; and (b) Chlamydia trachomatis (25) employs a different mechanism in which attachment to, and subsequent infectivity of, eukaryotic cells is dependent on a heparan sulfate-like ligand on the surface of the organism that bridges acceptors on the microorganism and host cell surface membranes, facilitating cell-cell contact. It has been proposed that the heparan sulfate-like adhesin is synthesized by Chlamydia (25). Our finding that low doses of soluble, sulfated polysaccharides induce a significant increase in tachyzoite infectivity suggests that the Toxoplasma receptor belongs to the second class of molecules. This was further supported by the fact that increasing doses of soluble proteoglycans and sulfated polysaccharides not only abolished such an increase, but at the highest concentration produced a significant inhibition, as expected when receptors on both cell surfaces are saturated, inhibiting the ternary complexes.

The availability of mutant cell lines with genetic defects in glycosaminoglycan assembly provides us with additional data to support the idea that sulfated polysaccharides participate in host cell infection. Our results with these cell lines indicate that heparan sulfate may be the host-derived ligand for the parasite lectin. However, heparin but not heparan sulfate was found to inhibit the lectin activity as assessed by inhibition of hemagglutination and modified infection of HFF cells. Because the structural analysis of the glycosaminoglycan chain of heparan sulfate has demonstrated the presence of heparin segments in its structure (45), we hypothesize that these heparin-like regions may mimic the natural carbohydrate ligand for the parasite lectin. This suggestion is further supported by the fact that preliminary results from experiments using glycosaminoglycan lysates indicated that treatment of HFF cells with heparinase (specific for heparin) but not heparitinase (specific for heparan sulfate) (45) or chondroitinase ABC resulted in about a 25% decrease in the ability of Toxoplasma to infect the host cell (data not shown). Taken together, the evidence presented supports the hypothesis that T. gondii utilizes cell surface sulfated sugars, including a heparin-like molecule, to interact with the host cell.

To gain insight into the identity of the Toxoplasma lectin activity, we exploited its ability to bind a variety of eukaryotic cells. First, by overlaying nitrocellulose filters, containing separated Toxoplasma proteins, with a suspension of red blood cells, we identified putative adhesins of 45, 65, and 71 kDa. The fact that preincubation of the filter with fucoidan abolished the binding of the 65- and 71-kDa proteins provided evidence of their sugar-binding ability (data not shown). We next asked whether intrinsically radiolabeled Toxoplasma proteins were able to bind to fixed cells and found that molecules of 26 and 65 kDa bound in a sugar inhibitable manner to the surface of the erythrocytes, whereas a protein of 45 kDa resisted elution by all the inhibitors tested. The relative participation of these proteins in the process of infection is currently unknown, but their diversity and specificity indicates that Toxoplasma, like other microorganisms, may employ various molecules to interact with the host.

Several groups have pursued the study of sugar-binding proteins on Toxoplasma (9, 46–48). Specific binding of neoglycoproteins (i.e. BSA-GlcN) to live tachyzoites was observed by Robert et al. (46). Lectins and neoglycoproteins (BSA-GlNaC and BSA-β-galactose) labeled with colloidal gold particles were used by de Carvalho et al. (47) to probe thin sections of tachyzoites with the result that significant labeling of the rhoptries was seen. In neither case, however, were the authors able to identify or determine the function of the parasite molecule responsible for the sugar-binding activity. In another study, inhibition of infection of fibroblast by preincubation of tachyzoites with BSA-GlcN was observed (9). Because this effect was significantly higher in a strain of T. gondii expressing SAG1 than in a SAG1-deficient mutant, Mineo et al. (9) concluded that SAG1 likely binds a glycosylated host cell receptor. More recently, a 15-kDa fetuin-binding protein was identified using affinity chromatography on fetuin-agarose (48). Differences in carbohydrate specificity, biological effect observed, and molecular mass distinguish these sugar-binding proteins from the sulfated polysaccharide-specific lectin(s) described in this paper. Determining the relative contribution of all these molecules to parasite infectivity is essential to a better understanding of the pathogenic process in toxoplasmosis. They also provide specificities and tentative identities (by size) for the lectins involved, making their further characterization now possible.

Acknowledgments—We thank Drs. Adrian Hебl, Ian Manger, and other members of this laboratory for helpful discussions and advice. We also thank Dr. David Sibley for exchange of information before publication.

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