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IDENTIFICATION AND PARTIAL PURIFICATION OF A LECTIN ON THE SURFACE OF THE SPOROZOITE OF *CRYPTOSPORIDIUM PARVUM*

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ABSTRACT: A human-derived isolate of *Cryptosporidium parvum* from a symptomatic patient with the acquired immunodeficiency syndrome was expanded in vivo by infecting a neonatal calf with 10^8 oocysts. Sporozoites were isolated from 4×10^{10} oocysts harvested from this single infection, and the characteristics of mixed hemagglutination (HA) with rabbit erythrocytes were determined. Sporozoite HA was inhibited by bovine submaxillary mucin (BSM), hog gastric mucin, and orosomucoid, but not by simple sugars, including sialic acid. Carbohydrate-inhibitable HA (lectin) activity increased with sporozoite lysis and was associated with the sporozoite membrane fractions. The ability of intact sporozoites to form rosettes around erythrocytes indicates that the HA (lectin) is, at least in part, present on the parasite surface. Hemagglutination (lectin) activity was partially purified from sporozoite lysates by affinity chromatography with BSM coupled to Sepharose-4B. Best elution was obtained with ethylene glycol and NaCl, which resulted in enrichment of 6 bands compared to the crude starting lysate (Mr = 60, 24, 22, 20, and 15 kDa and a 40-kDa doublet). Our results indicate that an HA (lectin) activity is present on the surface of intact sporozoites where it could play a role in cell-to-cell interactions with eukaryotic targets.

Cryptosporidium parvum is a sporozoan parasite that infects mucosal surfaces and produces diarrhea in some animals and more consistently in humans (Tzipori, 1988). In contrast to the usual self-limited diarrhea in normal humans (Tzipori et al., 1980; Isaacs et al., 1985; Wolfson et al., 1985), *C. parvum* produces either a severe acute dehydrating secretory diarrhea or a chronic diarrhea that leads to malabsorption and significant wasting in immunocompromised patients, especially those with acquired immunodeficiency syndrome (AIDS) (Malebranche et al., 1983; Cohen et al., 1984; Colebunders et al., 1987). Pathogenesis is poorly understood and no proven effective therapy exists (Tzipori, 1988).

Cryptosporidium parvum has a clear preference for certain sites within the host. In the human, the stomach rarely is infected, and the upper small bowel, colon, and rectum are less affected than the midsmall bowel (Tzipori, 1988). Animal studies indicate a similar prominence of infection of the ileum (Tzipori, 1988). In animals, susceptibility is also age dependent and apparently independent of immune status (Tzipori et al., 1982; Tzipori, 1988). These data suggest the possibility that developmentally regulated host factors, such as a surface receptor for the parasite, are involved in pathogenesis.

Infection is initiated by ingestion of the oocyst form, which excysts within the lumen of the small bowel. The released sporozoites invade the epithelial cell surface membrane (Tyzzer, 1910). Adherence of the parasite to the epithelial cell surface presumably precedes the invasive process, providing an opportunity for site-specific interactions to occur. The molecular basis of this postulated adherence and subsequent invasion of the cell by *C. parvum* is unknown. Membrane-bound sugar-binding proteins (or lectins), often stage specific, mediate cell–cell interactions in other protozoan parasites, such as *Plasmodium falciparum, Trypanosoma cruzi, Leishmania tropica, Entamoeba histolytica,* and *Giardia lamblia* (Chang, 1981; Crane and Dvorak, 1982; Vanderberg et al., 1985; Lev et al., 1986; Ravdin et al., 1989). Therefore, we searched for a surface lectin in the infective sporozoite form of *C. parvum.*

MATERIALS AND METHODS

Viable *C. parvum* oocysts were obtained from the stool of 2 symptomatic AIDS patients with severe diarrhea and purified by gradient centrifugation using discontinuous sucrose and isopyknic Percoll gradients as described by Arrowood and Sterling (1987). Oocysts (10^8) from 1 of these isolates were used to infect a neonatal calf. Approximately 4×10^{10} oocysts were

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TABLE I. Sugar hapten inhibitors tested for hemagglutination inhibition of oocysts of *Cryptosporidium parvum*.

Substance	Maximum concentration tested (mg/ml)	Inhibitory concentration (mg/ml)
Bovine submaxillary mucin	10	0.6
Hog gastric mucin	10	0.156
Asialofetuin	10	2.5
Orosomucoid	10	>10.0
Fetuin	200	>10.0
N-acetylglucosamine	100	>200.0
N-acetylgalactosamine	200	>200.0
D-Arabinoside	200	>200.0
D-Melibiose	200	>200.0
α-D-methyl-glucose	100	>100.0
D-Arabinose	200	>200.0
α-D-glucose	200	>200.0
D-Mannose	200	>200.0
D-N-acetylgalactosamine	200	>200.0
β-D-mannosamine	200	>200.0
Methyl-β-D-galactose	200	>200.0
Mannoside	200	>200.0
Methyl-α-mannoside	200	>200.0
Methyl-α-glucoside	200	>200.0
N-acetylneuramin-lactose	200	>200.0
N-acetyl-D-galactosamine	200	>200.0

TABLE II. Sugar hapten inhibitors tested for hemagglutination inhibition of sporozoites of *Cryptosporidium parvum*.

Substance	Maximum concentration tested (mg/ml)	Inhibitory concentration (mg/ml)
Bovine submaxillary mucin	10	0.015
Hog gastric mucin	10	0.156
Orosomucoid	10	2.5
Fetuin	10	>10.0
Asialofetuin	10	>10.0
Sialic acid	200	>200.00
Chitin	10	>10.0
Neuraminic acid	100	>100.0
N-acetylglucosamine	200	>200.0
α-D-methylglucose	200	>200.0
N-acetylgalactosamine	200	>200.0
D-Galactose	200	>200.0
D-Glucosamine	200	>200.0
L-Xylose	200	>200.0
Lactose	100	>100.0
D-Arabinoside	200	>200.0
L-Fucose	200	>200.0
D-Fucose	200	>200.0
L-Rhamnose	200	>200.0
D-Mannosamine	200	>200.0
D-Melibiose	200	>200.0
α-D-methylmannoside	100	>100.0
α-D-glucose	200	>200.0
D-Mannose	200	>200.0
D–N-acetylgalactosamine	200	>200.0
β-D–Mannosamine	200	>200.0
Methyl-β-D–galactose	200	>200.0

harvested from the diarrheic stool from this animal by the same method. To obtain sporozoites, oocysts were excysted in vitro by washing with 20 mM phosphate-buffered normal saline (PBS), pH 7.2, and then incubating in 0.5% trypsin and 1.5% taurocholate in PBS for 60 min at 37 C. Unexcysted oocysts, oocyst walls, and sporozoites were separated by gradient centrifugation on an isopyknic Percoll gradient (Arrowood and Sterling, 1987). Oocyst and sporozoite preparations used for these experiments were free of bacteria and debris by direct microscopic observations.

Erythrocytes from humans (groups A, B, and O), rabbits, rats, and mice were collected in sodium citrate and stored at 4 C in Alsiever's solution until used. For each experiment the erythrocytes were washed 3 times in PBS, suspended to a concentration of 2% in PBS, and kept at 4 C. Twenty-five microliters of the erythrocyte suspension were placed in each of 96 wells of a U-bottomed microtiter plate. When used, sporozoite lysate was prepared by washing approximately 10^9 Percoll-purified sporozoites 2 times in PBS (pH 7.2) and lysing them by ultrasonic disruption at 50% power output and 30% duty cycle of a Branson 450 sonifier in the presence of protease inhibitors (chymotrypsin, 2 μM; pepstatin, 5 μM; soybean trypsin inhibitor, 10 μg/ml) in RPMI medium containing HEPES, 5 μM, and iodoacetamide, 5 μM. Twenty-five microliters of a suspension of intact sporozoites or oocysts, or the sporozoite lysate adjusted to a concentration of 10^8 organisms/ml, then was added to the first well and serially diluted to a titer of 2,048. The hemagglutination (HA) plate was incubated at 4 C for 40 min and read. Each experiment was replicated 3 times per plate. The highest dilution of intact organisms or lysate giving a visible HA reaction was defined as the HA titer. Erythrocytes from each of the host species tested were fixed in 1.0% glutaraldehyde in PBS for 2 hr at 4 C and compared with unfixed erythrocytes from the same species in the HA reaction. Minor differences were seen in HA activity with erythrocytes from different hosts whether fixed or not. Due to greater stability and consistency, glutaraldehyde-fixed erythrocytes from rabbits were chosen for all HA inhibition and rosette experiments. The optimal conditions for HA were selected by individually varying pH, temperature, and divalent cation concentration. For pH determination, the lysate, erythrocytes, and diluent were dialyzed to equilibrium against sodium barbital buffer at a pH varying by 0.5 units from 4 to 10. Hemagglutination was compared at 4 C, room temperature, and 37 C. The requirement for Ca^{2+} and Mg^{2+} was determined by making doubling serial dilutions of saturated solutions of ethylendiaminotetraacetic acid (EDTA) and ethylene glycoltetraacetic acid (EGTA) to 4 HA units of sporozoite or lysate in the microtiter system prior to the addition of erythrocytes. To determine HA-inhibition titers, doubling serial dilutions of soluble hapten inhibitors (see Tables I, II) were added to the wells of microtiter plates containing 4 HA units of organisms or lysate and a 2% suspension of glutaraldehyde-fixed rabbit erythrocytes. The minimum concentration of inhibitor preventing visible HA was defined as the inhibitory concentration. Carbohydrate-inhibitable HA was considered to be evidence of lectin activity.

Sporozoite rosette formation was demonstrated by incubating approximately 10^6 sporozoites and 25 μl of a 2% suspension of rabbit erythrocytes at room temperature for 30 min in a U-bottomed microtiter plate.

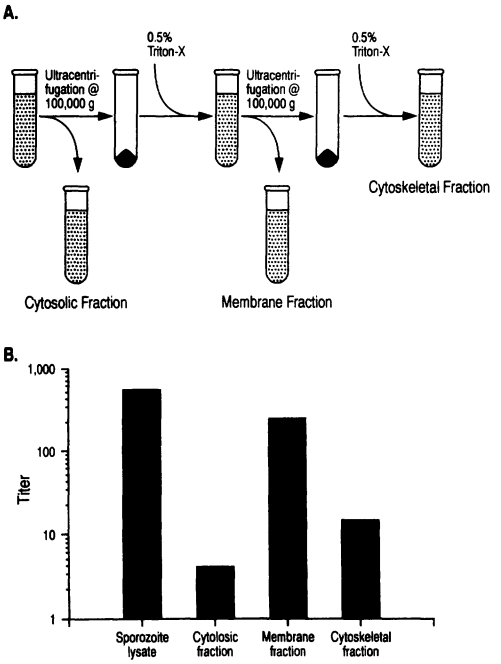

FIGURE 1. Membrane fractionation scheme (A) and hemagglutination titers (B) of sporozoite membrane and cytoskeletal and cytosolic fractions obtained by differential centrifugation following lysis and extraction of 10^9 sporozoites (see Materials and Methods). Titers were determined in equal concentrations of each preparation.

The suspension was transferred gently to a cytocentrifuge (Shandon Southern Instruments, Inc., Sewickly, Pennsylvania) for sedimentation onto a microscope slide and then fixed in methanol and stained with Diff-Quik.

Crude lysate from 10^9 freshly excysted sporozoites was prepared as indicated above and centrifuged at 100,000 *g* at 4 C for 120 min (Fig. 1). The supernatant portion was removed and saved as the cytosolic fraction. The pellet was resuspended in an equal volume of 0.5% Triton X-100 in PBS without protease inhibitors and left at 4 C overnight. This was spun at 100,000 *g* again for 120 min and the supernatant fraction was saved as the membrane fraction. The pellet then was resuspended in an equal volume of 0.5% Triton X-100 as the cytoskeletal fraction. Equal volumes of these 3 crude preparations were assayed for HA activity.

Bovine submaxillary mucin (Boehringer Mannheim, Indianapolis, Indiana, catalogue number 102 032) was coupled to cyanogen bromide-activated Sepharose-CL-4B-200 (Sigma Chemical Co., St. Louis, Missouri) as previously described (March et al., 1974). Lysate, prepared by ultrasonic disruption of 10^9 sporozoites in 3 ml of 0.5% Triton X-100 in PBS, was centrifuged in a Microfuge to remove particulate debris and passed through a 0.45-μM Millex HV_{13} Millipore filter. The clarified lysate was mixed with 5 ml of bovine submaxillary mucin (BSM)-coupled Sepharose-4B in a 50-ml centrifuge tube and allowed to equilibrate overnight

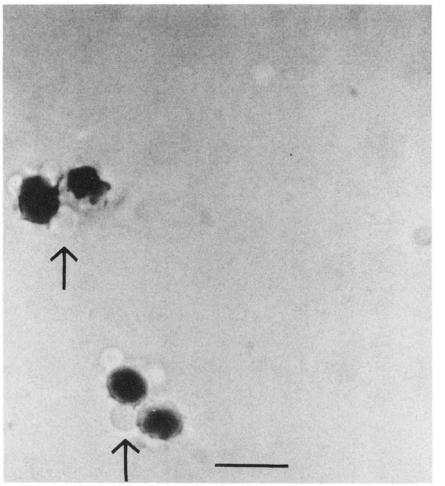

FIGURE 2. *Cryptosporidium parvum* oocyst rosette formation (arrows) around glutaraldehyde-fixed rabbit erythrocytes. Scale bar = 14 μm.

at 4 C with gentle rocking. The mixture was poured into a 1.0 × 5.0-cm glass column and washed with 4 bed volumes of PBS. It then was eluted sequentially with 3 ml of 50% ethylene glycol, 0.5 M, 1.0 M, 1.5 M, 2 M, 2.5 M, and 3 M NaCl, and 1-ml elution fractions were collected and analyzed for HA activity.

HeLa cells were grown to near confluency in RPMI containing 10% fetal calf serum and antibiotics in 8-chamber Micro-tek tissue culture slide chambers. Growth medium was aspirated from the chambers and replaced with PBS containing 10^6 oocysts. Parasites were added and allowed to settle and attach to the monolayer for 30 min at room temperature. The monolayer was washed gently 3 times with PBS, stained with Giemsa solution, and observed microscopically. Attachment inhibition was evaluated by suspending a companion aliquot of oocysts in PBS containing 100 mg/ml of hog gastric mucin prior to addition to the monolayers and performing the assay as described above.

RESULTS

Hemagglutination was detected by a mixed agglutination test in 2 *C. parvum* isolates. A concentration of 10^8 oocysts in both preparations produced a titer of 1:16. The oocyst lectin was active with human A, B, and O, mouse, sheep, and rabbit erythrocytes, whether native or glutaraldehyde-treated. For uniformity and convenience, glutaraldehyde-fixed rabbit erythrocytes were used in subsequent studies. Both isolates formed oocyst rosettes around erythrocytes (Fig. 2). The HA titer increased 4-fold, from 1:16 to 1:256, when 10^8 oocysts first were lysed by ultrasonication. Among 22 sugar haptens tested as possible HA inhibitors, only 3 (hog gastric mucin

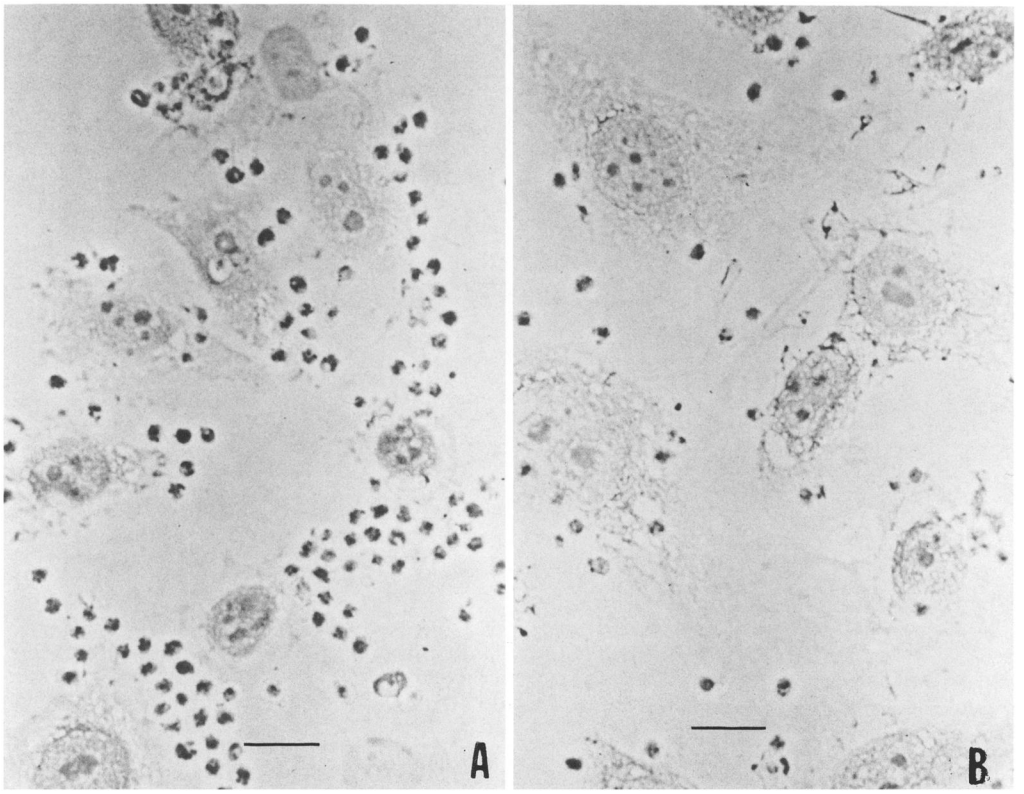

FIGURE 3. Attachment of *Cryptosporidium parvum* oocysts to HeLa cells in monolayer culture. **A.** Attachment in the absence of inhibitory glycoproteins. **B.** Inhibition of attachment in the presence of 100 μg/ml of hog gastric mucin. Scale bar = 20 μm.

[HGM], BSM, and asialofetuin) were active (Table I), as determined in the mixed HA assay. Oocysts of both isolates adhered to the surface of HeLa cells in monolayer culture (Fig. 3A); adherence was inhibited partially by addition of 100 μg/ml HGM (Fig. 3B).

One isolate, DK-1, was expanded by experimentally infecting a neonatal calf. Four × 10^{10} oocysts were recovered from the pooled diarrheal stool of this animal, and all further experiments were conducted with this isolate. Like oocysts, sporozoites reacted with rabbit, mouse, sheep, and human A, B, and O erythrocytes. Again, the

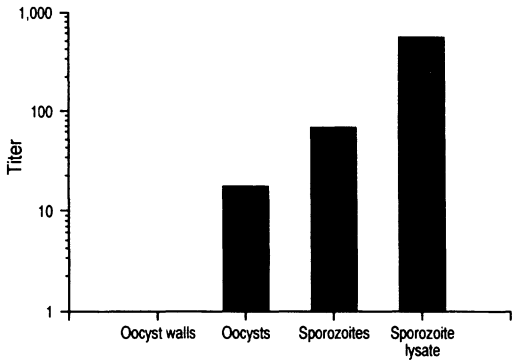

FIGURE 4. Titers of mixed hemagglutination assay using glutaraldehyde-fixed rabbit erythrocytes and 10^8 intact oocysts, excysted sporozoites, sporozoite lysate, and oocyst walls.

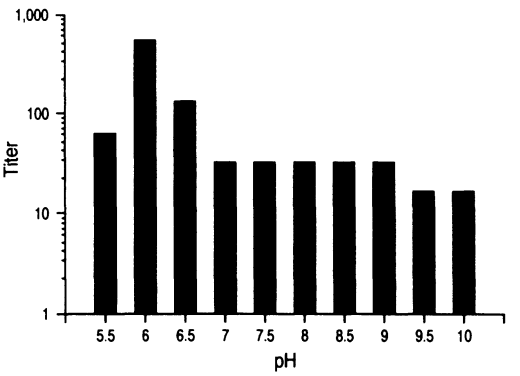

FIGURE 5. Effect of pH on hemagglutination titer of sporozoite lysate.

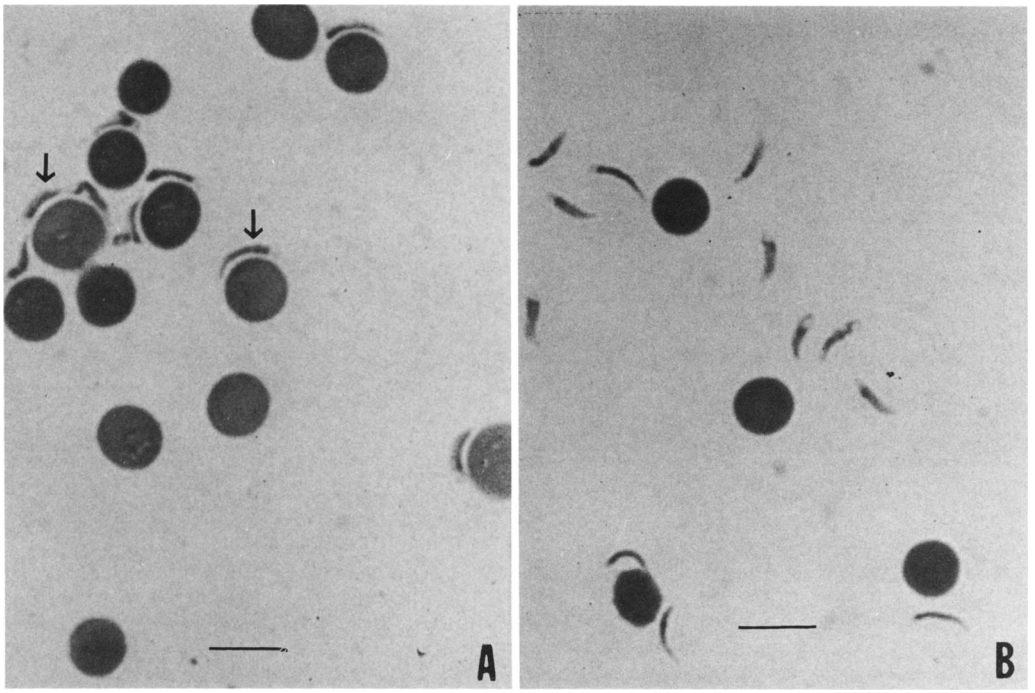

FIGURE 6. Rosette formation of *Cryptosporidium parvum* sporozoites (arrows) with glutaraldehyde-fixed rabbit erythrocytes. **A.** Rosette formation in the absence of inhibitory glycoproteins. **B.** Inhibition of rosette formation in the presence of 100 μg/ml of hog gastric mucin. Scale bar = 10 μm.

strongest reactions were present with glutaraldehyde-fixed rabbit erythrocytes, which were used for all subsequent assays. Excysted viable sporozoites exhibited a 2-fold increase in HA activity in the mixed HA assay, compared with an equivalent number of unexcysted oocysts. In contrast, free oocyst walls harvested after excystation lacked HA activity (Fig. 4).

Sporozoite lysates, prepared by ultrasonic disruption, gave a further 3-fold increase in HA titer compared with intact sporozoites (Fig. 4). The lysate therefore was used for studies to characterize the lectin further and to determine optimal conditions for HA. Hemagglutination assays performed at 4 C exhibited a 2–3-fold increase in titer compared to 25 C or 37 C. Divalent cations were not required for HA and the titer was not affected by EDTA or EGTA. The pH optimum was 6.0 (Fig. 5); however, reliable data could not be obtained below pH 5.5 because of nonspecific agglutination in all wells. Maximum lectin activity was present in whole sporozoite lysate containing cytosolic and membrane fractions. After separation, the membrane fraction exhibited 4-fold greater activity than the cytoskeletal and 5-fold greater activity than the cytosolic fractions (Fig. 1). Of the 28 simple sugars, oligosaccharides, and glycoproteins tested for their ability to inhibit sporozoite HA activity (Table II), only 3 glycoproteins: BSM, HGM, and orosomucoid, inhibited the HA reaction. The most effective inhibitor was BSM, which was 10 times more active on a weight basis than HGM. In contrast to observations with the intact oocysts, asialofetuin did not inhibit the sporozoite HA. Mixed sporozoite–erythrocyte aggregates formed when freshly excysted whole sporozoites were added to glutaraldehyde-fixed rabbit erythrocytes (Fig. 6A). Aggregate formation was inhibited (Fig. 6B) in the presence of 100 μg/ml of BSM.

Partial purification of lectin activity was achieved by affinity chromatography on BSM coupled to Sepharose-4B. Application of 3,084 HA units to the column resulted in the recovery of 770 HA units after washing with PBS and elution with 50% ethylene glycol and increasing concentrations of NaCl. We were unable to find conditions for specific elution of HA activity using soluble sugar haptens. Ethylene glycol and NaCl fractions containing the peak HA activity

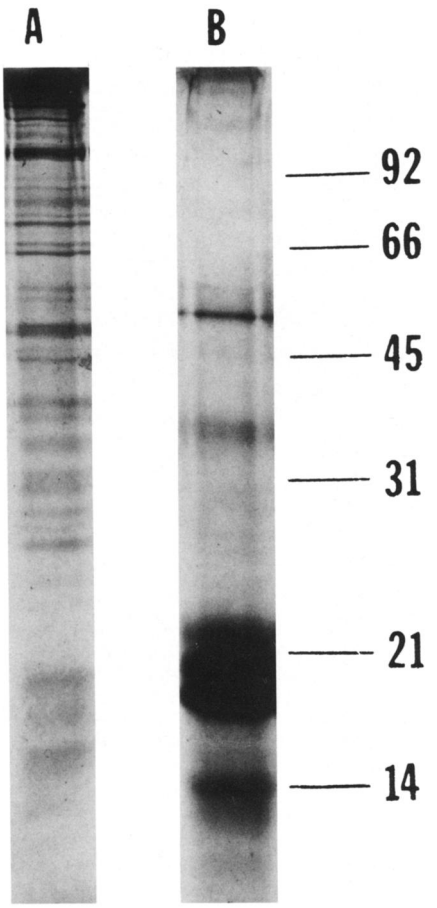

FIGURE 7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of crude sporozoite lysate (A) and bovine submaxillary mucin–Sepharose affinity column pooled eluate containing peak hemagglutinating activity (B). Molecular weights (kDa) are indicated.

were pooled and concentrated 200× by lyophilization after dialysis against 20 mM ammonium bicarbonate. This then was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and developed using a silver stain. The number of protein bands was reduced sharply compared to the crude starting lysate, and 6 bands (Mr = 60, 24, 22, 20, and 15 kDa, and a 40-kDa doublet) were enriched (Fig. 7).

DISCUSSION

We have demonstrated mixed hemagglutinating activity in 2 isolates of *C. parvum* isolated from the stool of human AIDS patients with diarrhea. Hemagglutination was expressed preferentially on the invasive sporozoite stage. Although intact oocysts resulted in a mixed agglutination at a low titer with glutaraldehyde-fixed rabbit erythrocytes, the HA activity remained associated with the sporozoites when intact oocysts and empty oocyst walls were separated by differential centrifugation. It is possible, therefore, that the oocyst resident HA activity is lost or inactivated during excystation. Intact sporozoites also formed clearcut rosettes around erythrocytes, suggesting that the binding constituent was present on the parasite surface membrane.

These cell–cell interactions were inhibitable by several complex carbohydrates, indicating the lectin nature of the HA. Mucins containing complex sugars, including bovine submaxillary mucin and hog gastric mucin, were effective inhibitors but not the simple or substituted mono- and disaccharides tested. Bovine submaxiillary mucin was a 166-fold more potent inhibitor of sporozoite–erythrocyte binding than orosomucoid. This pattern of lectin activity is similar to phytohemagglutinin and the lectin from *Euonymus europeus* reported by Petryniak et al. (1977), which are sensitive to low concentrations of polysaccharides and glycoproteins but not to high concentrations of mono- and disaccharides. The sporozoite mixed-HA was also inhibited to a lesser extent by orosomucoid as was oocyst HA by asialofetuin. Further sugar specificities are being sought. Except for asialofetuin, all of the complex glycoproteins showing inhibitory activity are known to be rich in terminal sialic acid residues. Bovine submaxillary mucin is particularly rich in these residues and is abundant on the surface of mammalian cells. However, sialic acid alone was not inhibitory to either lectin activity as measured by the HA assay, suggesting that if sialic acid is important in the observed adherence of parasite to erythrocyte, it is in the form of a more complex oligosaccharide or a substituted neuraminic acid moiety. Desialylated fetuin, asialofetuin, showed weak inhibitory activity suggesting that the exposed terminal (N-acetylglucosamine)$_2$-galactose residues may also be part of the binding site. The optimum pH for HA was slightly acidic (6.0), which is consistent with the pH at the intestinal mucosal surface (Rhodes and Prestwich, 1966). This is also identical to the pH optimum reported by Ward et al. (1987) for HA activity of the lectin of *G. lamblia,* another parasite that attaches to the small intestinal epithelial cell brush-border membrane.

We were able to solubilize the sporozoite HA in 0.5% Triton X-100 and subsequently recov-

ered lectin activity in the 100,000-*g* supernatant fraction (membrane fraction). The solubilized HA was purified partially by affinity chromatography on BSM coupled to Sepharose-4B although sugar-specific elution was not accomplished. The pooled and concentrated lectin eluate was enriched in 6 protein bands detected by SDS-PAGE ranging in size from 14 to 60 kDa. It is not known whether any of these proteins represent the HA itself. One candidate is the 23-kDa band, as other workers recently have described a surface-located protein of similar molecular mass that induces a serological response in infected humans and animals (Ungar and Nash, 1986; Mead et al., 1988). A similar-sized antigen also has been demonstrated by immunoelectron microscopy on the external surface of the pellicle organelles at the anterior end of both invasive stages of *C. parvum,* sporozoites and merozoites (Lamb et al., 1989). This would be consistent also with other data suggesting that polar organelles are involved directly in invasion by parasites in the phylum Apicomplexa (Russell, 1983).

Our results indicate that sugar-inhibitable HA is present on the surface of the intact oocyst as well as on the sporozoite. If lectins are involved in cell–cell interactions of the parasite in the intestinal tract, their presence on the oocyst might serve to localize this stage in regions of the intestine subject to invasion by the sporozoite. Subsequent local release of sporozoites from attached oocysts might actually increase the efficiency of invasion. If the HA activity in free oocyst walls truly decreased after excystation, this would serve to minimize competition with infective oocysts at the mucosal border, another strategy that increases the efficiency of sporozoite attachment and subsequent invasion.

However, it should be stated clearly that it is not yet known whether the HA activity we have demonstrated has any physiological relevance. If the lectin is important in pathogenesis, and is present in differing amounts on invasive parasite stages in the intestinal lumen, then local secretory antibody could inhibit infection. Monoclonal antibodies to 20- and 28-kDa sporozoite proteins have been reported to be protective in a murine infection model (Riggs and Perryman, 1987; Arrowood et al., 1989). We do not know if these antibodies neutralize the HA, or whether they recognize any of the bands we detect in the partially purified lectin. The 20-kDa antigen recognized by 1 of these antibodies may be the same as the previously reported immunodominant 23 kDa antigen from sonicated oocysts (Ungar and Nash, 1986). Hyperimmune colostral antibody preparations have been used with some apparent success for passive antibody therapy in human AIDS patients (Nord et al., 1990). It is not yet known whether these contain anti-lectin activity. If they do, the possibility that high-titered anti-lectin antibody may improve the response to passive immunotherapy of intractable cryptosporidiosis would be increased.

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