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Characterization of the *Cryptosporidium* Antigens from Sporulated Oocysts of *Cryptosporidium parvum*

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The antigenic constituents of sporulated *Cryptosporidium parvum* oocyst antigens were characterized with antisera from mice immunized against *C. parvum*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining defined the major proteins. Six of seven lectins used recognized as many as 15 bands. The lectins concanavalin A, *Dolichos biflorus*, and wheat germ agglutinin showed strong activity against the same eight bands with molecular weights ranging from 72,000 to greater than 100,000. An enzyme-linked immunosorbent assay was used to detect antibody to *C. parvum*. Antibody binding was significantly decreased by heat and enzymatic treatment with trypsin, protease, and mixed glycosidases. *C. parvum* antigens were further defined by the reactivity of immune sera with a *C. parvum* sonicate preparation separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose paper. Antisera from orally infected mice consistently recognized four antigens with molecular weights ranging from 72,000 to greater than 100,000. These antigens also bound concanavalin A. Treatment of the antigen preparation with mixed glycosidases reduced the reactivity of antisera with most antigens with molecular weights greater than 60,000. The data suggest that the antigenic composition of *C. parvum* is complex and that carbohydrates alone or in association with lipids or proteins may be important in the immune response to *C. parvum*.

Cryptosporidium parvum is a significant cause of diarrhea in domesticated animals (1, 3) and in both immunocompetent and immunocompromised patients (4, 18). The role of humoral immunity in the development of resistance is not known, although antibody against *C. parvum* has been demonstrated in the sera of infected normal (2, 13, 16) and infected immunodeficient (16) individuals. In this study, we have characterized the antibody response of mice infected with *C. parvum* and determined the contribution of carbohydrates to the antigenicity of sporulated *C. parvum* oocysts. These studies may prove valuable for the further study of host-parasite interactions and for the development of newer diagnostic methods and vaccines.

MATERIALS AND METHODS

***C. parvum* antigens.** *C. parvum* oocysts, originally isolated from a calf, were collected from the feces of infected calves as previously described (7). Feces suspended in phosphate-buffered saline (PBS) (pH 7.2) were treated with ethyl acetate (Sigma Chemical Co., St. Louis, Mo.) at a 1:10 dilution to remove fecal fat and centrifuged at 350 × *g*. The pellet was washed twice in distilled water. The preparation was layered over a discontinuous sucrose gradient of three densities, 1.18, 1.09, and 1.02 g/ml, and centrifuged at 450 × *g* for 20 min. A broad band of oocysts contaminated with bacteria were found within the 1.09-g/ml density of sucrose. The contaminating bacteria were removed by placing the oocyst suspension in a stirred cell fitted with a 3-μm-pore-size polycarbonate membrane filter (Nuclepore Corp., Pleasanton, Calif.). With adequate stirring, bacteria passed through the filter, and the oocyst suspension was microscopically free of bacteria and fecal debris. The suspension was centrifuged at 500 × *g*, the supernatant was removed, and the pellet was suspended at 2 × 10^7 oocysts per ml of PBS.

In preparations of oocysts to be sonicated for antigen preparations used in polyacrylamide gel electrophoresis (PAGE) and Western blotting (immunoblotting), the following proteolytic inhibitors were added to the oocyst suspension prior to sonication: 20 mM *N*-ethylmaleimide, 20 mM EDTA, 20 mM benzamide hydrochloride, 10 mM merasyl acid, 10 μM D-phenyl-alamyl-L-arginine chloromethyl ketone, 50 μg of leupeptin per ml, and 50 μg of L-chloro-3-(tosylamine)-7-amino-2-hepatone hydrochloride (Sigma) per ml.

The oocyst suspension was sonicated with a Biosonic sonicator 15 to 20 times (18,000 Hz) at 20-s intervals over 60 min at 4°C. Greater than 95% of the oocysts were disrupted. Undisrupted oocysts were removed by centrifuging the oocyst suspension at 400 × *g* for 10 min. The supernatant was divided into aliquots and stored at −70°C.

Antigen preparations were separated by ultracentrifugation of *C. parvum* sonic extracts. Antigen preparations were centrifuged at 100,000 × *g* for 2 h at 4°C. The supernatant was separated and used as a source of soluble antigens, and the pellets were washed twice in PBS and used as insoluble pellet antigens containing the surface membrane, organelles, and nucleus (9, 12). In experiments in which the antigenic reactivity of the soluble fraction and that of the insoluble pellet were compared, antigens derived from equivalent numbers of oocysts were added to each lane.

ELISA of anti-*C. parvum* antibody and enzyme treatment of antigen on plates. The immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) was performed essentially as previously described (9) but modified for *C. parvum* antigen. Briefly, sonicated antigen preparations from 10^5 *C. parvum* oocysts that were diluted in 0.1 M carbonate buffer (pH 9.8) and that had been either heat treated (100°C for 1 h) or not treated were dispensed in 100-μl aliquots into Linbro

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polyvinyl chloride microfiltration plates (Flow Laboratories, Inc., McLean, Va.) and incubated overnight at 4°C. The plates were washed with PBS (pH 7.2) containing 0.05% Tween 20 three times for 5 min each time. Excess binding sites on the wells were blocked by postcoating the wells with 250 μl of PBS containing 5% bovine serum albumin (BSA) and 0.05% Tween 20 for 2 h at 37°C. The plates were washed as described above. Sera diluted in PBS containing 5% BSA were added to each well in triplicate in a volume of 100 μl and incubated for 1 h at 37°C. The plates were washed, alkaline phosphatase-conjugated rabbit anti-mouse IgG (specific) antibody (Bio-Rad Laboratories, Hercules, Calif.) diluted in PBS–5% BSA–0.05% Tween 20 was added in a volume of 100 μl per well, and the plates were incubated for 1 h at 37°C. After the plates were washed, 100 μl of *p*-nitrophenyl phosphate (Sigma) was added at a concentration of 0.66 mg/ml in 0.15 M sodium bicarbonate containing 1 mM $MgCl_2$, and the plates were incubated at room temperature for 3 h. The A_{405} was determined with an ELISA reader (Biotek Instruments, Winooski, Vt.).

In experiments in which antigens were enzymatically treated on microtiter plates, the antigen was applied to the plate by being dried overnight at 37°C and was then treated enzymatically as described by Ramasamy and Reese (10). The bound antigen was treated with 200 μl of trypsin at 1,100 U/ml, protease at 20 U/ml, or neuraminidase at 2 U/ml (Sigma) in PBS for 30 min at 37°C. After incubation, the protease and neuraminidase reactions were stopped by the addition of cold (4°C) PBS. Trypsin was neutralized by adding soybean trypsin inhibitor (Sigma) at a concentration of 500 μg/ml for 15 min. Antigens were also treated with mixed glycosidases from *Charonic lampas* (Miles Laboratories, Inc., Elkhart, Ind.) at 20 mg/ml in 0.01 M sodium acetate (pH 4.0) for 20 min at 37°C. To minimize the effect of any contaminating proteolytic enzymes, we coincubated various proteolytic enzyme inhibitors, including 20 mM EDTA, 50 μg of leupeptin per ml, and 10 μg of aprotinin per ml, with the mixed glycosidases. After enzymatic treatment, the ELISA was performed as described above. Antigens incubated in parallel without enzymes were used as controls. In preliminary experiments, treatment of antigens bound to culture plates with mixed glycosidases in this manner did not significantly diminish the amount of plate-bound protein when compared with untreated controls. Furthermore, treatment of antigens with mixed glycosidases decreased the binding of concanavalin A to the treated antigens by over 90% when compared with untreated antigens.

PAGE of proteins. Sonicated parasite preparations were dissolved in a sample buffer that contained 0.1 M Tris hydrochloride, 3% sodium dodecyl sulfate (SDS), 5% glycerol, 10 mM EDTA, 0.001% bromophenol blue, and either 2.5% mercaptoethanol or no mercaptoethanol and were boiled for 3 min. Samples were electrophoresed in a discontinuous 0.1% SDS–10% polyacrylamide gel on a Mini gel apparatus (Bio-Rad) with the buffers of Laemmli (6) to reduce the amount of sample needed. Phosphorylase *b*, BSA, ovalbumin, chymotrypsinogen, β-lactoglobulin, and lysozyme (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used as molecular weight standards.

Silver staining. After electrophoresis, the polyacrylamide gels were soaked overnight in 40% ethanol–5% acetic acid. The gels were washed three times in distilled water. Silver stain freshly prepared by the method of Wray et al. (17) was used to stain the gels. Briefly, the gels were stained in a solution of 0.8% silver nitrate in 0.02 M sodium hydroxide–0.2 M ammonium hydroxide and agitated vigorously for 10 min. The gels were washed for 5 min in distilled water and developed in a solution of 0.005% citric acid and 0.019% formaldehyde. The gels were washed three times in distilled water.

Electrophoretic transfer of proteins. Proteins separated by one-dimensional SDS gel electrophoresis were transferred to 0.45-μm-pore-size nitrocellulose paper as described by Towbin et al. (14). Excess protein-binding sites were blocked with 5% BSA in Tris-saline (0.9% NaCl, 10 mM Tris hydrochloride [pH 7.4]) for 2 h at room temperature. The blots were washed in Tris-saline three times and incubated overnight with control serum or serum containing anti-*C. parvum* antibodies diluted to a concentration of 1:20 or 1:50 in 5% BSA. The blots were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (Bio-Rad) for 2 h at room temperature. The blots were washed and developed in a solution of 0.02% diaminobenzidine, 0.1% hydrogen peroxide, and 0.01 M PBS. The color development was stopped by washing the blots with Tris-saline after 20 min of incubation. Dried nitrocellulose blots were photographed immediately.

Detection of lectin-binding proteins. Proteins of *C. parvum* sonicate were separated on 10% SDS gels and electrophoretically transferred to nitrocellulose paper as described above. The blots were incubated with biotinylated lectins (concanavalin A, *Ricinus communis* agglutinin, *Dolichos biflorus* agglutinin, wheat germ agglutinin, soybean agglutinin, *Ulex europeas* agglutinin I, and peanut agglutinin) (Vector Laboratories, South San Francisco, Calif.), diluted in PBS–5% BSA at a concentration of 10 to 20 μg/ml, and incubated overnight. To test the specificity of the lectins for particular carbohydrate moieties, we incubated the lectins with or without specific sugars, including α-methyl mannoside (100 mM), β-D-lactose (100 mM), *N*-acetylgalactosamine (100 mM), *N*-acetylglucosamine (50 mM), D-galactose (50 mM), L-fucose (100 mM), and D-galactose (200 mM) (Sigma), respectively. The blots were washed several times with Tris-saline and incubated for 2 h with avidin biotinylated to horseradish peroxidase. The blots were washed and developed in a solution of 0.02% diaminobenzidine, 0.1% hydrogen peroxide, and 0.01 M PBS. The color development was stopped by washing the blots with Tris saline after 10 min of incubation. Dried nitrocellulose blots were photographed immediately.

Sera. Sera were pooled from three groups of 10 6-week-old Swiss Webster mice (Taconic Farms, Germantown, N.Y.). Mice were infected with 10^4 *C. parvum* oocysts by gavage at 3 days of age and then weekly thereafter for 4 weeks. Three pools of sera were obtained 3 weeks after the last gavage and were designated O_1, O_2, and O_3. In preliminary experiments, we demonstrated that oral infections initiated in the neonatal period resulted in persistent infections for 10 to 14 days. Immune sera were also pooled from 10 Swiss-Webster female mice that had received four weekly intraperitoneal injections of 10^4 *C. parvum* oocysts and were designated IP_1 and IP_2.

RESULTS

***C. parvum* proteins and glycoproteins.** The silver-stained proteins of a sonic extract of sporulated *C. parvum* oocysts and sporozoites are shown in Fig. 1. Biotinylated lectins were used to detect which polypeptide bands were glycoproteins. Various lectins were used to detect various glycoproteins of *C. parvum* oocysts and sporozoites. The results of the glycoprotein analysis are shown in Fig. 2. Six

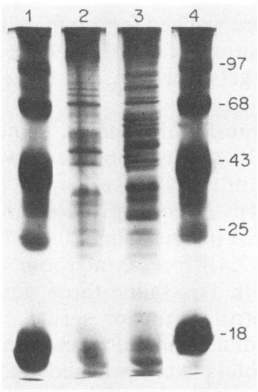

FIG. 1. SDS-PAGE of *C. parvum* oocyst and sporozoite proteins separated on 10% SDS–PAGE slab gels. Protein preparations were either not reduced (lane 2) or reduced (lane 3) with 2-mercaptoethanol prior to electrophoresis. Molecular weight standards are shown in lanes 1 and 4 and labeled (in thousands) in the right-hand margin.

TABLE 1. Effect of various enzymatic and heat treatments on the antigenicity of *C. parvum* antigens for antibodies from orally infected mice[a]

Antigen treatment	A_{405} (mean ± SD)
Enzymatic	
None (untreated control)	0.735 ± 0.02
Mixed glycosidases	0.502 ± 0.01[b]
Trypsin	0.240 ± 0.01[c]
Protease	0.185 ± 0.01[c]
Neuraminidase	0.691 ± 0.003[b]
Heat	
None (untreated control)	0.535 ± 0.02
Heat (100°C)	0.177 ± 0.03[c]

[a] Sera were obtained from mice that had received four doses of 10^4 *C. parvum* oocysts by gavage at weekly intervals. Student's *t* test was used as a test of the significance between the reactivity of the treated and untreated antigen preparations.
[b] Significant at $P < 0.05$.
[c] Significant at $P < 0.01$.

of the seven lectins used recognized as many as 15 bands and, in each instance, the lectin binding to the protein could be inhibited by coincubation of the particular lectin with a simple sugar specific for that lectin. In addition, because this system uses avidin biotinylated to horseradish peroxidase, we also demonstrated that the avidin biotinylated to horseradish peroxidase itself did not bind to the polypeptide bands (data not shown). The complexity of the carbohydrates was further demonstrated in that concanavalin A, *D. biflorus* agglutinin, and wheat germ agglutinin showed strong activity against the same eight bands with apparent molecular weights of 72,000, 81,000, 87,000, 92,000, 95,000, and 99,000 and two major bands with a molecular weight greater than 100,000. The specificity and avidity of each of these lectins for these polypeptides and the ability of relatively low concentrations of specific simple sugars to inhibit the binding of these lectins to these polypeptides suggest that the two carbohydrate components are complex with α-D-mannosyl and/or α-D-glucosyl and/or *N*-acetyl-α-D-galactosaminyl terminal residues and that they contain *N*-acetyl-β-D-glycosaminyl residues and/or *N*-acetyl-β-glucosamine oligomers.

Effects of heat and enzymatic treatments on the antigenicity of *C. parvum* antigens in an IgG ELISA. To characterize the antigens that were recognized by murine IgG antibodies produced in response to infection with *C. parvum*, we measured, in an IgG ELISA, the reactivity of sera obtained from either orally or intraperitoneally infected mice with *C. parvum* antigen which had been either enzymatically or heat treated or not treated at all. Heat denaturation of the antigen preparation, which had a more profound effect on proteins than on carbohydrates, significantly decreased but did not eliminate the reactivity of sera from orally (Table 1) and intraperitoneally (Table 2) infected mice. Denaturation by heat did not have as profound an effect on the activity of antibodies from mice infected intraperitoneally (20 to 25% decrease in reactivity) relative to orally infected mice (75% decrease in reactivity). Treatment of the antigen with the

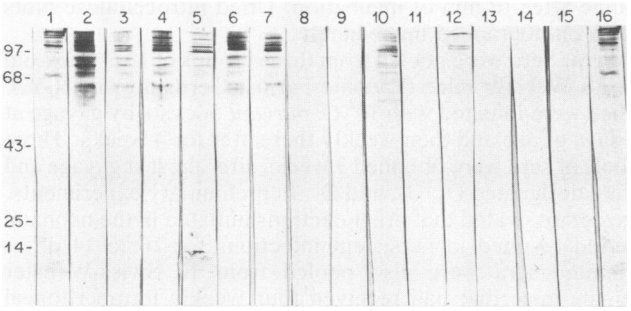

FIG. 2. Glycoprotein analysis of *C. parvum* oocyst and sporozoite proteins by various lectins, including concanavalin A (lane 2), *D. biflorus* agglutinin (lane 4), wheat germ agglutinin (lane 6), *U. europeas* agglutinin I (lane 8), *R. communis* agglutinin (lane 10), soybean agglutinin (lane 12), and peanut agglutinin (lane 14), without or with the specific sugars which inhibit lectin binding. These sugars included α-methyl mannoside (lane 3), β-D-lactose (lane 5), *N*-acetylgalactosamine (lane 7), *N*-acetylglucosamine (lane 9), D-galactose (lane 11), L-fucose (lane 13), and D-galactose (lane 15). Lanes 1 and 16 show the detection of *C. parvum* oocyst and sporozoite antigens by murine IgG from orally infected (O_1) mice. Numbers in the left-hand margin are molecular weights (in thousands).

TABLE 2. Effect of various enzymatic and heat treatments on the antigenicity of *C. parvum* antigens for antibodies from intraperitoneally inoculated mice[a]

Antigen treatment	A_{405} (mean ± SD) at indicated antibody titer		
	1:20	1:20	1:80
Enzymatic			
None (untreated control)	1.001 ± 0.04	1.155 ± 0.02	0.965 ± 0.037
Mixed glycosidases	0.630 ± 0.03[b]	0.741 ± 0.02[b]	0.569 ± 0.02[b]
Trypsin	0.348 ± 0.003[b]	0.319 ± 0.003[b]	0.211 ± 0.004[b]
Protease	0.130 ± 0.01[b]	0.064 ± 0.01[b]	0.025 ± 0.008[b]
Neuraminidase	0.896 ± 0.03[c]	0.912 ± 0.01[c]	0.767 ± 0.03[c]
Heat			
None (untreated control)	1.076 ± 0.02	1.076 ± 0.03	0.831 ± 0.03
Heat (100°C)	0.811 ± 0.04[c]	0.751 ± 0.02[b]	0.595 ± 0.02[b]

[a] Sera were obtained from mice that had received four intraperitoneal injections of 10^6 *C. parvum* oocysts at weekly intervals. Student's *t* test was used as a test of the significance between the reactivity of the treated and untreated antigen preparations.
[b] Significant at $P < 0.01$.
[c] Significant at $P < 0.05$.

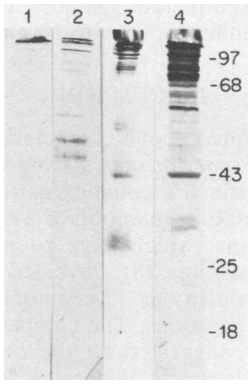

FIG. 3. Immunodetection of *C. parvum* antigens by antibodies of sera from mice either orally (O_2) (lanes 1 and 2) or intraperitoneally (IP_1) (lanes 3 and 4) inoculated with *C. parvum* oocysts. Antigen preparations were either reduced (lanes 2 and 4) or not reduced (lanes 1 and 3) with 2-mercaptoethanol. Molecular weight standards are shown (in thousands) in the right-hand margin.

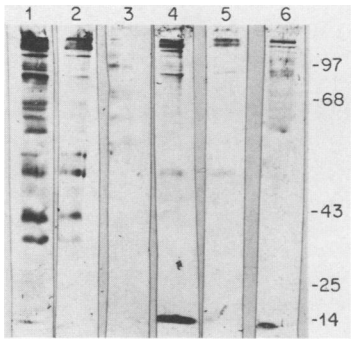

FIG. 4. Immunodetection of *C. parvum* antigens by antibodies of sera from mice orally (O_3) inoculated with *C. parvum* oocysts. Antigen preparations were either reduced (lanes 1 to 3) or not reduced (lanes 4 to 6) with 2-mercaptoethanol. *C. parvum* antigens were either not separated (lanes 1 and 4) or separated into insoluble (lanes 2 and 5) or soluble (lanes 3 and 6) fractions by ultracentrifugation. Molecular weight standards are shown (in thousands) in the right-hand margin.

proteolytic enzymes trypsin and protease profoundly decreased the reactivity of immune sera. These results indicate that, although proteolytic enzymes have a dramatic effect on antibody reactivity with *C. parvum* antigen, considerable reactivity is maintained despite heat denaturation. Furthermore, the mode of infection may affect the antigens being recognized.

To determine whether the oligosaccharides which we demonstrated in our lectin-binding studies may be important antigenic moieties, we used mixed glycosidases from *C. lampas* to cleave the oligosaccharide chains. The mixed glycosidases contained α- and β-mannosidases, α- and β-glucosidases, α- and β-galactosidases, α-L-fucosidase, β-D-xylosidase, α- and β-*N*-acetylglucosaminidases, and α- and β-*N*-acetylgalactosaminidases. Treatment of *C. parvum* sonicate with mixed glycosidases significantly ($P < 0.01$) decreased the reactivity of sera from both orally and intraperitoneally infected mice; however, the decrease in the reactivity of sera from intraperitoneally infected mice was more dramatic than that of sera from orally infected mice. To minimize the specificity of the glycosidase treatment of the antigen, we added protease inhibitors at the time of the glycosidase treatment to minimize the activity of possible contaminating proteolytic enzymes in our glycosidase preparation. In addition, the binding of concanavalin A to *C. parvum* sonicate was ablated after glycosidase treatment, suggesting the specificity of the enzyme treatment. Neuraminidase, which has activity against sialic acid residues, decreased the reactivity of sera from mice infected intraperitoneally (Table 2) with *C. parvum* antigen but did not remarkably affect the reactivity of sera from orally infected mice. Thus, proteins and carbohydrates of *C. parvum* appear to be imporatnt antigenic moieties.

***C. parvum* antigens detected by immune sera.** To further characterize the antibody response of mice infected with *C. parvum* to parasite proteins, a *C. parvum* sonicate preparation was separated by SDS-PAGE, electrophoretically transferred to nitroceullose paper, and reacted with sera pooled from several groups of mice infected orally or intraperitoneally with *C. parvum*. Representative results of experiments in which pooled sera from three different groups of mice that were infected by gavage with *C. parvum* were used are shown in Fig. 2 (lanes 1 and 16), 3 (lanes 1 and 2), 4, and 5 (lane 3). The antigen preparations were either reduced or not reduced with 2-mercaptoethanol. When nonreduced antigens were used, reactivity with two high-molecular-weight antigens (greater than 100,000) was consistently demonstrated (Fig. 3, lane 1, and Fig. 4, lane 4). There was variability in the number of bands recognized by different pools of sera; however, as many as seven bands with molecular weights ranging from 55,000 to greater than 100,000 were recognized by sera from orally infected mice (Fig. 4, lane 4). Upon reduction of the antigens with 2-mercaptoethanol, antigens with molecular weights of 72,000 to 76,000, 98,000, and greater than 100,000 (two antigens) were consistently recognized by sera from orally infected mice (Fig. 2, lane 1, Fig. 3, lane 2, Fig. 4, lane 1, and Fig. 5, lane 3). These antigens also bound concanavalin A and other lectins (Fig. 2), indicating that they are glycoproteins. Sera from mice inoculated intraperitoneally recognized the same antigens as well as a more extensive array of antigens (Fig. 3, lanes 3 and 4, Fig. 5, lane 1, and Fig. 6). When sera from

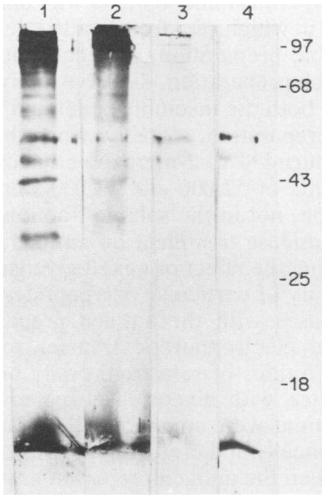

FIG. 5. Immunodetection of *C. parvum* antigens either not treated (lanes 1 and 3) or treated (lanes 2 and 4) with mixed glycosidases. Immune sera used were from mice infected either intraperitoneally (IP_2) or orally (O_1). Molecular weight standards are shown (in thousands) in the right-hand margin.

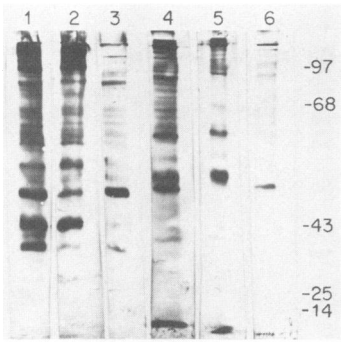

FIG. 6. Immunodetection of *C. parvum* antigens by antibodies of sera from mice intraperitoneally (IP_2) inoculated with *C. parvum* oocysts. Antigen preparations were either reduced (lanes 1 to 3) or not reduced (lanes 4 to 6) with 2-mercaptoethanol. *C. parvum* antigens were either not separated (lanes 1 and 4) or separated into insoluble (lanes 2 and 5) or soluble (lanes 3 and 6) fractions by ultracentrifugation. Molecular weight standards are shown (in thousands) in the right-hand margin.

uninfected mice were used as the primary antibody or when the secondary antibody was used alone, no bands were recognized.

To determine whether the antigens recognized by immune sera are found on the insoluble pellet or soluble *C. parvum* fraction, we subjected a *C. parvum* sonicate preparation to differential centrifugation. Preparations from the two fractions were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose paper, and the reactivities of sera from orally infected and intraperitoneally inoculated mice were assessed. The reactivities of antisera from orally and intraperitoneally infected mice with the subcellular fractions are shown in Fig. 4 and 6, respectively. The reactivities of antisera from orally infected mice (Fig. 4, lanes 2 and 5) and intraperitoneally inoculated mice (Fig. 6, lanes 2 and 5) with the insoluble pellet appeared to be similar to those with the major antigens of the noncentrifuged whole-antigen preparation. However, the intensity of the reactivity of numerous antigens with apparent molecular weights between 55,000 and 100,000 was diminished, especially in studies in which sera from orally infected mice were used. The soluble preparation, although not as reactive as the whole-antigen preparation, did have many antigens identical to those in both the insoluble pellet and noncentrifuged whole-antigen preparation. Notably, when the antigen preparation was reduced with 2-mercaptoethanol, the bands at molecular weights of 57,000 and 43,000 were found in the insoluble fraction, not in the soluble fraction.

Effect of glycosidase treatment on antibody reactivity. To directly determine the effect of mixed-glycosidase treatment on the antigenicity of particular polypeptides, we incubated mixed glycosidases with the antigen preparation prior to SDS-PAGE and electrophoretic transfer to nitrocellulose paper. The reactivities of sera from orally or intraperitoneally infected mice with glycosidase-treated and untreated antigen preparations were compared. The reactivities of sera from intraperitoneally and orally infected mice were remarkably reduced when the antigen preparation was treated with mixed glycosidases (Fig. 5). Reactivities with almost all antigens with molecular weights above 60,000, except perhaps the 98,000-molecular-weight antigen which was demonstrated with sera from intraperitoneally inoculated mice, were markedly reduced. These data further support the contention that carbohydrate moieties may be important antigens in the immune response to *C. parvum*.

DISCUSSION

The results presented above characterize certain properties of antigens of *C. parvum* oocysts and sporozoites. In the initial studies we defined a complex pattern of proteins by silver staining a PAGE preparation of a sonic extract of *C. parvum*. Plant lectins, which have been used as specific probes for carbohydrates (8), revealed the presence of carbohydrates in as many as 15 components present in *C. parvum* antigen preparations. The complexity of the carbohydrate moieties is suggested by the observation that as many as eight of these components avidly bound concanavalin A. Each of the lectins is specific for particular oligosaccharides, and lectin binding could be specifically inhibited in the presence of low concentrations of competing saccharides. Furthermore, five of the seven lectins used bound to protein bands. Most likely, the lectins interact with specific glycoproteins, although there is a possibility that glycolipids may be involved. A comparison of the protein bands recognized by antibodies from animals orally infected with *C. parvum* with those that bound concanavalin A revealed that both the antibodies and the lectin reacted with proteins of similar molecular weights and, in particular, reacted strongly with the 98,000-molecular-weight antigen. This result suggested that the carbohydrate moieties may be involved in the immune response.

To further characterize the biochemical nature of the antigens recognized by the antibodies of immune mice, we subjected a *C. parvum* sonicate preparation to heat and enzymatic digestion. Treatment of the antigen with protease, trypsin, or heat at 100°C decreased the reactivity of the antigen preparation with antibodies from mice either orally or intraperitoneally infected with *C. parvum*. Heat treatment had a more dramatic effect on decreasing the reactivity with sera from orally infected mice relative to intraperitoneally infected mice. Heat treatment, which has a more profound effect on proteins than on carbohydrates, did not ablate the reactivity of the antigen with immune sera. We therefore found it of interest to determine the effect of cleavage of the carbohydrate moieties from glycoproteins by mixed glycosidases on the antigenicity of *C. parvum*. Removal of the oligosaccharides or sialic acid by mixed glycosidases or neuraminidase significantly reduced the antigenicity of *C. parvum* for sera from orally and intraperitoneally infected mice, respectively. In contrast to heat treatment, mixed-glycosidase treatment had a more dramatic effect on the reactivity of sera from intraperitoneally infected mice relative to orally infected mice. These data suggest that the antigenicities of protein and carbohydrate epitopes may be dependent on the mode of infection. Although these data suggest that oligosaccharide side chains of glycoproteins of *C. parvum* are potentially antigenic, they do not address the possibility that cleavage of the oligosaccharides may have altered the tertiary and quaternary structure of the parasite proteins, thus reducing the ability of the antibodies to bind to particular antigenic epitopes.

The immunologic significance of the various constituents of the *C. parvum* sporulated oocyst preparation was explored by Western blot analyses. In these analyses, sera from five groups of mice, three orally infected and two intraperitoneally inoculated, were used to assess the major antigenic components of *C. parvum*. Although there was variability in the antigens recognized by the different pools

of sera, common bands were recognized by all pools of sera from orally infected mice. Sera from intraperitoneally inoculated mice recognized similar antigens as well as numerous other antigenic determinants. Numerous investigators have used differential centrifugation to separate membrane-associated (insoluble pellet) from cytoplasmic (soluble) antigenic components of bacteria (5, 11) and protozoa (9, 12). Using this technique, we found that although the intensity of binding of immune sera was greater toward the insoluble pellet fraction than toward the soluble fraction, there were many bands of identity between these two fractions. Only two antigens with apparent molecular weights of 43,000 and 57,000 were recognized by sera from immunized mice and found to be unique to the membrane-associated fraction. Recently, Ungar and Nash (15) reported that a 23,000-molecular-weight antigen was commonly recognized by sera of humans infected with *C. parvum*. In no instance did sera from immunized mice recognize this low-molecular-weight antigen. However, they also mentioned in their report that three high-molecular-weight antigens (greater than 125,000) were detected in 50% of patients with cryptosporidiosis. These antigens may be similar to the high-molecular-weight antigens detected by the sera of our immunized mice.

Western blot analysis was also used to determine which antigenic determinant was diminished by glycosidase treatment of the parasite antigen preparation. Glycosidase treatment decreased the reactivity of various antigens with molecular weights above 60,000, suggesting further that carbohydrate moieties are important immunogens in *C. parvum* infections. Future studies regarding the use of recombinant purified proteins for serological tests or vaccines may need to consider the role of carbohydrates in the immune response to this pathogen.

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