Inhibition of *Giardia lamblia* Excystation by Antibodies against Cyst Walls and by Wheat Germ Agglutinin

TZE-CHIANG MENG,¹ MICHAEL L. HETSKO,² AND FRANCES D. GILLIN^{2,3*}

Division of Infectious Diseases, Department of Medicine,¹ Department of Pathology,² and Center for Molecular Genetics,³ University of California at San Diego Medical Center, San Diego, California 92103-8416

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Although excystation is crucial to the initiation of infection by *Giardia lamblia*, little is known about the regulation of this important process. We have been able to reliably induce excystation in vitro by mimicking cyst passage through the stomach and upper small intestine by the exposure of in vitro-derived cysts to an acidic, reducing environment (stage I) followed by protease treatment at a slightly alkaline pH (stage II). Preexposure of cysts to polyclonal rabbit antiserum against purified cyst walls (PCWs) or to wheat germ agglutinin (WGA) inhibited excystation by >90%. Adsorption of either ligand with PCWs eliminated inhibition, demonstrating specificity for cyst wall epitopes. Inhibition by WGA was reversed by either chitotriose or sialic acid, while inhibition by polyclonal antibodies against PCWs (anti-PCW) was reversed only by sialic acid, which also inhibited binding of both ligands to intact cysts and to cyst wall antigens in immunoblots. Binding of anti-PCW did not affect acidification of cyst cytoplasm during stage I. Exposure of cysts to anti-PCW and WGA prior to, but not after, stage II was sufficient to inhibit excystation, and inhibition could be partially reversed by increasing the protease concentration during stage II. A 7- to 10-fold higher proportion of WGA- and anti-PCW-treated cysts than control cysts remained intact after stage II. Our results suggest that these ligands, which bind cyst wall epitopes, inhibit excystation, most likely by interfering with proteolysis of cyst wall glycoproteins during stage II.

Giardia lamblia is a major cause of waterborne gastrointestinal disease in the United States and of malnutrition from malabsorption worldwide (35). The trophozoite form that colonizes the human intestinal tract is capable of differentiating into a resistant cyst in response to appropriate stimuli (1). Cysts are absolutely required for survival outside the host, as well as for initiating new infections (28). Ingested cysts undergo excystation in the upper small intestine of the new host, releasing the disease-causing trophozoites (1). In prior studies (4), we completed the life cycle of *G. lamblia* in vitro, demonstrating for the first time that excystation of in vitro-derived cysts, like that of fecal cysts (3, 29), could be accomplished by mimicking passage through the acid-filled stomach and into the protease-rich upper small intestine. Since this critical differentiation process is very poorly understood, we have begun to dissect the mechanism(s) of excystation.

The cyst wall must be sufficiently impervious to protect the parasite within from the rigors of both the external environment and passage through the stomach but must also permit response to the external signals triggering excystation. Purified cyst wall structures (PCWs), which morphologically resemble the wall of intact cysts, are produced by boiling cysts in sodium dodecyl sulfate (SDS) (11). Chemical analyses suggest the primary structural component of PCWs is a polymer of galactosamine (GalN) and/or *N*-acetylgalactosamine (GalNAc) (11). Under reducing conditions, several proteins and glycoproteins, some containing *N*-acetylglucosamine (GlcNAc) (21, 25), are released from PCWs (26). Recently genes encoding two leucine-rich cyst wall proteins have been identified (14, 18).

In our prior studies on expression and transport of cyst wall

antigens, we identified two groups of cyst antigens (10, 24, 26). Group I antigens consist of several broad protein bands, ~26 to 46 kDa, that are expressed beginning within ~3 h of encystation and are each recognized by the monoclonal antibodies (MAbs) GCSA-1 and 8C5 (5, 26, 34). Immunoelectron microscopy demonstrated that these epitopes localize to the interior of the encystation-specific secretory vesicles and to the cyst wall (16, 27) and also appear to be recognized by polyclonal antibodies against PCWs (anti-PCW). Group II antigens consist of at least four glycoprotein bands, ~66, 85, 120, and 140 kDa, that localize to the cyst wall and are recognized by wheat germ agglutinin (WGA) (24, 25), which is specific for (GlcNAc)_n or sialic acid, as well by anti-PCW serum. In previous investigations of host immune responses, we found that many patients with giardiasis have serum and/or mucosal antibodies which preferentially recognize these WGA-binding cyst wall glycoproteins (25).

These findings prompted us to initiate the first studies of the mechanism of excystation of *G. lamblia* in vitro by examining whether antibodies and lectins that bind to the outside of the cyst wall could interfere with this differentiation process.

MATERIALS AND METHODS

Materials. Unless otherwise specified, reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.). Bicarbonate was purchased from Fisher Scientific Co. (Pittsburgh, Pa.). Adult bovine serum was purchased from HyClone (Logan, Utah).

Cultivation of parasites. *G. lamblia* trophozoites (strain WB, ATCC 30957, clone C6) were routinely cultivated at 37°C in Diamond TYI-S-33 medium containing 10% adult bovine serum (7), pH 7.1, supplemented with bovine bile (12) (0.5 mg/ml) but without added iron, vitamins, or antibiotics. Trophozoites were subcultured twice weekly.

Preparation of cysts in vitro. Cultures grown to late-log phase were chilled and trophozoites were enumerated in a hemacytometer. Trophozoites (3,000/ml, final concentration) were added to freshly prepared pre-encystation medium (4): TYI-S-33 medium containing 10% adult bovine serum, pH 7.1, without bile. After 72 h, the attached monolayer (≥60% confluent) was refed with encystation medium: TYI-S-33 medium containing 10% adult bovine serum, adjusted to pH 7.8 with 1 M NaOH, and supplemented with lactic acid (hemi-calcium salt, 5

* Corresponding author. Mailing address: UCSD Medical Center, Department of Pathology, 214 Dickinson St., CTF-403C, San Diego, CA 92103-8416. Phone: (619) 543-6146. Fax: (619) 543-6614. Electronic mail address: fgillin@ucsd.edu.

mM), porcine bile (0.25 mg/ml), piperacillin (500 µg/ml; Lederle Laboratories, Carolina, P.R.), and amikacin (125 µg/ml). After approximately 66 h, the medium containing the nonattached population, including the cysts, was removed, and the cells were harvested by centrifugation at $2,200 \times g$ for 5 min at room temperature. Trophozoites and incomplete cysts were lysed by incubation in double-distilled water for 20 min at room temperature. After several low speed ($1,140 \times g$) washes at 4°C, water-resistant cysts were stored in double-distilled water at 4°C overnight, prior to excystation.

Excystation of in vitro-derived cysts. In vitro-derived cysts were enumerated on the day of excystation in a hemacytometer chamber, and viability was determined by diluting cysts in an equal volume of 0.4% trypan blue (Gibco-BRL, Gaithersville, Md.). The percentage of type I cysts, which are smooth, slightly refractile, oval bodies with the organelles of fecal cysts and are more likely to be viable (8), was determined by differential interference contrast microscopy. Unless otherwise specified, excystation was induced by a modification of the two-step method of Rice and Schaefer (4, 29). Stage I solution was freshly made by mixing 6.8 ml of Hanks buffered salt solution (pH 6.8) containing L-cysteine (57 mM) and reduced glutathione (32.5 mM) with 6.8 ml of 0.1 M NaHCO₃ and 11.36 ml of water and then adjusting the pH to 4.0 with HCl. Cysts (1.5×10^6 total, unless otherwise specified) were pelleted at $8,300 \times g$ at 4°C, resuspended in 550 µl of prewarmed stage I solution, and incubated for 20 min at 37°C. Cysts were then pelleted and resuspended in 1 ml of prewarmed stage II solution (Tyrodes buffered salt solution [pH adjusted to 8.0 with 7.5% NaHCO₃] with 1-mg/ml bovine trypsin type II) for 1 h at 37°C. Cysts were then pelleted at $8,300 \times g$ and resuspended in 100 µl of cultivation medium for 30 min at 37°C (stage III). The emerged motile trophozoites were enumerated by a hemacytometer. The percent excystation was calculated as the sum of the motile trophozoites and partially emerged trophozoites divided by the initial number of viable type I cysts.

Antibodies against cyst wall antigens. PCWs were obtained by boiling water-resistant in vitro-derived cysts in 1% SDS for 10 to 15 min (11). New Zealand White rabbits were immunized intravenously with 10^6 PCWs without adjuvant, twice weekly for five immunizations (26). Complement was inactivated by incubation of serum for 1 h at 56°C prior to use. MAb GCSA-1 was a gift from H. Ward (34) and MAb 8C5 was a gift from G. Faubert (5).

Inhibition of excystation. Unless otherwise stated, in vitro-derived cysts were preincubated prior to stage I in 0.1 to 0.4 ml of diluted antiserum (10% in water) or lectin diluted in lectin solution (73 mM NaCl, 100 mM MgCl₂, 100 mM CaCl₂) for 1 h at 4°C. Preincubation in water, preimmune serum, or lectin solution, respectively, was used as a control to calculate percent inhibition. After determining reliable inhibitory concentrations, specificity of the inhibition was determined by adsorption and sugar competition. Reduced PCWs were produced by boiling in vitro-derived cysts in 1% SDS for 5 min in the presence of 70 mM dithiothreitol. Anti-PCW serum (10%) was adsorbed against PCWs or reduced PCWs (final concentration, 5×10^7 /ml) overnight at 4°C on a rocker. Initial experiments indicated that overnight incubation of WGA resulted in loss of inhibitory activity; therefore, WGA (10 µg/ml) was incubated with PCWs or reduced PCWs for 1 h at 4°C immediately prior to preincubation with the cysts. Competition for binding was evaluated by adding the sugar to diluted antibody or lectin before the addition of cysts. Unbound antibodies, lectin, or sugar was removed by pelleting the cysts at $8,300 \times g$ for 5 min at 4°C. Stability of binding was evaluated by preincubating cysts with anti-PCW serum or WGA, removing unbound antibody or WGA, and then incubating the treated cysts with sialic acid or chitotriose. The effect of the timing of exposure was evaluated by varying the stage at which the cysts were preincubated with anti-PCW serum or WGA.

The efficiency of excystation varied among experiments, as would be expected of a complex differentiation process. Therefore, the mean inhibition by various ligands was normalized to the respective control for each experiment. All experiments were repeated at least twice. *P* values were calculated by paired two-tailed *t* test. Data shown are means $\pm$ standard deviations for at least two independent experiments. To calculate the mean reversal of inhibition by competing sugars, the results of each experiment were normalized to the maximal inhibition by antibody or WGA for that experiment. *P* values were calculated by paired two-tailed *t* test with the normalized comparison value set at 100% for control excystation or 0% for maximal inhibition.

Measurement of cyst cytosol pH. Cysts were resuspended to 10^7 /ml and incubated with intracellular pH indicator 2',7'-bis-(2-carboxyethyl)-5 (and-6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM) (5 mM; Molecular Probes, Eugene, Oreg.) (30) in labeling buffer (pH 6.9; 10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid [HEPES], 130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose) at 37°C for 30 min. Excess label was removed by washing three times with labeling buffer, and then cysts were preincubated with antibody (10%) as described above. After washing the cysts three times with water, 100 µl of cyst suspension (1.6×10^7 to 2.0×10^7 /ml) was added to 1.9 ml of the target pH buffer (pH_{out}). Emission at 535 nm was determined with excitation at 505 and 439 nm by using a Perkin-Elmer LS3 fluorescence spectrophotometer. Internal pH (pH_{in}) was determined by interpolation from a standard curve of the 535 nm emission ratio, with excitation at 505 and 439 nm, of labeled cysts in high potassium pH buffers [pH range 4.0 to 7.8; 125 mM KCl, 1.2 mM KH₂PO₄, 1 mM MgSO₄, 1.2 mM CaCl₂; buffered with 10 mM sodium acetate for pH 4.0 and 5.0; 10 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES) for pH 6.0 and 6.4; or 10 mM HEPES for pH 6.8, 7.2, and 7.8] in the presence of the potassium

FIG. 1. Effects of anti-PCW serum and lectins on excystation. Percent excystation is compared with preimmune serum or lectin solution, respectively. The means $\pm$ one standard deviation of two experiments are shown. Cysts were preincubated in diluted serum or lectin prior to stage I of excystation. *, *P* < 0.05, paired two-tailed *t* test, compared with preimmune serum or lectin solution controls; Con A, concanavalin A.

ionophore nigericin (10 mM; Molecular Probes), which equalizes pH_{in} and pH_{out} (32).

Preparation of immunoblots. Cyst antigens for replicate immunoblots were prepared by scaling up excystations 5- to 10-fold, maintaining the ratio of cyst number to stage I and stage II solution volumes as previously described. Cells were washed three times in water in the presence of 1 mM phenylmethylsulfonyl fluoride and 1 mM trans-epoxysuccinyl-L-leucyl(4-guanidino)-butane (E64). Cells were resuspended to a concentration of 3×10^8 /ml in SDS sample buffer, with or without dithiothreitol (35 mM final concentration), and boiled for ~5 min. Proteins were separated by SDS-10% polyacrylamide gel electrophoresis (13) and transferred to nitrocellulose (33). After blocking for 1 h, blots were incubated in antibody diluted in gelatin buffer (25) (1:100) or WGA-horseradish peroxidase (HRP) diluted in lectin solution (0.5 µg/ml) for 1 h. Goat anti-rabbit immunoglobulin G alkaline phosphatase (1:3,000) was utilized to localize the rabbit polyclonal antibodies. To evaluate whether sugars block recognition of specific cyst wall epitopes, anti-PCW serum (1.0%) and WGA-HRP (20 µg/ml) were preincubated with chitotriose or sialic acid (50 and 100 mM, respectively) for 30 min at 4°C prior to addition to immunoblots of PCW antigens.

Immunocytochemistry. Cysts were incubated with anti-PCW serum (10%), WGA-HRP (1:2,000), or their respective controls, with or without prior incubation of ligand with chitotriose or sialic acid (50 mM), for 1 h at 4°C. Antiserum-treated cysts were washed three times with ice-cold phosphate-buffered saline (PBS) and then incubated with anti-rabbit immunoglobulin G-HRP (1:500) for 1 h at 4°C. After washing, cysts were developed in substrate (0.5-mg/ml 4-chloro-1-naphthol, 16.7% [vol/vol] methanol, and 0.015% [vol/vol] H₂O₂ in PBS) and observed under differential interference contrast microscopy (24).

RESULTS

Inhibition of excystation. Preincubation of in vitro-derived cysts in anti-PCW serum at a concentration of 10% consistently inhibited excystation >90% (Fig. 1), while preincubation in preimmune serum did not. To determine which of the antigens recognized by anti-PCW serum were involved in inhibition, we tested MAb GCSA-1 and 8C5, which are specific for the group I antigens, and WGA, which is specific for the group II antigens. Preincubation of cysts in the MABs had no appar-

TABLE 1. Blockage of inhibition by increasing cyst numbers or by adsorption with PCWs or reduced PCWs

Exptl variable ^a	% Excystation with indicated variable ^b		% Inhibition
	Preimmune	Anti-PCW	
No. of cysts			
1 × 10 ⁶	70.1	4.1	94.2
5 × 10 ⁶	86.4	41.2	52.3
1 × 10 ⁷	45.4	35.1	22.7
	Preimmune	Anti-PCW	
Anti-PCW			
Nonadsorbed	54.7	18.7	65.8
PCW adsorbed	56.7	54.7	3.5
Reduced PCW adsorbed	57.3	54.0	5.8
	Lectin solution	WGA	
WGA			
Nonadsorbed	50.7	15.3	69.8
PCW adsorbed		54.7	0
Reduced PCW adsorbed		54.0	0

^a Increasing cyst number during preincubation (1 ml constant volume of 10% anti-PCW) reduced the inhibitory effect, suggesting the inhibitory factor could be titrated out. The percent excystation of controls also appeared to decrease with increasing cyst concentrations, possibly because of decreased efficiency of proteolysis during stage II. Adsorption of anti-PCW serum (10%) or WGA (10 µg/ml) with PCWs or reduced PCWs (5 × 10⁷ per ml of diluted serum or lectin) prior to preincubation with cysts (4.5 × 10⁵ per condition) reversed the inhibition of excystation, indicating specific binding to PCW epitope(s).

^b Absolute percent excystation.

ent inhibitory effect, with maximal inhibition of ~20 and ~7% for MAbs GCSA-1 and 8C5, respectively, at ascites concentrations of 25%. WGA, on the other hand, consistently inhibited excystation at a concentration of 10 µg/ml (Fig. 1). This suggests that the group II glycoprotein antigens, rather than the group I antigens, are involved in the inhibition of excystation, although the lack of effect of the MAbs may be secondary to limited avidity or a narrow range of epitope recognition. Inhibition was proportional to the concentrations of WGA (Fig. 1) and anti-PCW (inhibition at 5% was less than 10% [data not shown]). Preincubation of cysts in WGA or antibody did not affect cyst viability, as determined by trypan blue exclusion (data not shown; see also below).

As WGA recognizes oligomers of D-N-acetylglucosamine (GlcNAc)_n or N-acetylneuraminic acid, we evaluated several other lectins to determine if inhibition of excystation was specific to certain carbohydrate epitopes. *Lycopersicon esculentum* lectin, which binds (GlcNAc)₃, recognized the group II antigens on immunoblots (data not shown) but did not reliably inhibit excystation. *Phaseolus limensis* lectin, which recognizes GalNAc and has been reported to bind to cysts (11), failed to inhibit excystation. Concanavalin A, which recognizes α-D-mannose or α-D-glucose and binds an 85-kDa protein on immunoblots of cyst antigens (23), also failed to inhibit excystation (Fig. 1); however, this epitope does not appear to be exposed on the external cyst surface. *Datura stramonium* lectin (which recognizes [GalNAc]₂) and *Tetragonolobus purpureus* lectin (which recognizes α-L-fucose) have not been shown to bind to cysts and did not inhibit excystation (data not shown).

Reversal of inhibition. Inhibition of excystation by anti-PCW serum was overcome by increasing the concentration of cysts (Table 1), suggesting that the inhibitory factor could be titrated or adsorbed out. Specificity of the inhibition to antibodies directed against cyst wall epitopes was shown by abrogation of inhibition when anti-PCW serum (10%) was adsorbed with

PCWs (Table 1) prior to excystation. Since boiling PCWs in reduced sample buffer released many proteins and glycoproteins, as determined by immunoblot, we also adsorbed anti-PCW serum with reduced PCWs prior to excystation. Surprisingly, inhibition was also abrogated by reduced PCWs (Table 1), suggesting that the ligand(s) associated with inhibition was either strongly attached to the cyst walls or intrinsic to the cyst wall. Not unexpectedly, inhibition by WGA was also eliminated by adsorption with PCWs or reduced PCWs (Table 1).

WGA-mediated inhibition of excystation was efficiently blocked by preincubation with triacetylchitotriose (GlcNAc)₃ or sialic acid (10 mM) (Fig. 2), probably through competition for WGA binding. Interestingly, the inhibitory action of anti-PCW serum was prevented by coinubation with sialic acid but not by triacetylchitotriose or other sugars (Fig. 2). Importantly, lack of competition by glucuronic acid supports the specificity for sialic acid.

Moreover, binding of anti-PCW and WGA to cysts was reversible. Addition of sialic acid, but not chitotriose, after cysts were preincubated with anti-PCW reversed the inhibition of excystation by anti-PCW serum, presumably by displacing bound ligand; both sugars reversed inhibition by WGA (data not shown). This also demonstrated that binding by WGA or anti-PCW serum did not kill the cysts. The sugars alone did not affect excystation at the same concentrations (data not shown). Immunocytochemistry demonstrated that intact cysts were recognized by anti-PCW serum but not by preimmune serum. This recognition was blocked by the addition of sialic acid but not by chitotriose (Fig. 3). Interestingly, preincubation of anti-PCW serum and WGA with sialic acid, but not chitotriose, abrogated recognition of cyst wall epitopes on immunoblots (Fig. 4). These results suggest that the binding epitope(s) for the inhibitory antibodies, like that for WGA, is a carbohydrate-containing moiety of the cyst wall.

Stage of inhibition by anti-PCW serum and WGA. In order to further evaluate the mechanism of inhibition by anti-PCW or WGA, we examined the effects of ligand binding on the individual steps of in vitro excystation. Stage I of excystation primarily mimics passage of the cyst through the acid-filled stomach (3), while stage II primarily mimics passage of the cyst into the protease-rich, slightly alkaline environment of the small intestine, distal to the entry of the common bile duct (29). To determine if antibody binding interferes with the physiologic response to external acidification or alkalization, we measured internal cyst pH as a function of external pH in the presence and absence of bound anti-PCW or preimmune serum. While mean pH_[in] decreased by 0.83 ± 0.48 pH units when cysts were placed in stage I solution (pH 4), no differences were found between cysts preincubated in anti-PCW serum (mean decrease, 0.90 ± 0.51 pH units) or preimmune serum (mean decrease, 0.70 ± 0.38 pH units). Antibody-treated and control cysts also had similar cytoplasmic pH responses to mild external alkalization (data not shown).

We next examined whether antigens recognized by anti-PCW serum or by WGA change during excystation and if this was affected by the presence of bound ligand. We compared immunoblots of cysts preincubated with preimmune serum, anti-PCW serum, or WGA before excystation, after stage I and after stage II. The patterns of the antigens recognized by either anti-PCW serum or WGA were altered after both stage I and stage II. However, no major differences were observed among cysts pretreated with preimmune serum, anti-PCW serum, or WGA (data not shown).

Although there was little difference in immunoblots, differential interference contrast microscopy revealed that cysts preincubated with either ligand appeared much less degraded

FIG. 2. Percent reversal of (A) WGA- or (B) anti-PCW-serum-mediated inhibition of excystation by coincubation with the indicated sugar concentration (mM). Values are the means $\pm$ one standard deviation of at least two separate experiments with each sugar, and the reversal of inhibition is normalized to the maximal inhibition by WGA and anti-PCW serum for the corresponding experiments. *, $P < 0.05$, paired two-tailed t test, compared with WGA (10 μ g/ml) or anti-PCW serum (10%).

than their respective controls after stage II (Fig. 5). A greater proportion of treated cysts retained the smooth, refractile, oval appearance of intact cysts (WGA $7.7\% \pm 2.5\%$ versus control $1.1\% \pm 1.5\%$, $P = 0.12$; anti-PCW $9.3\% \pm 2.2\%$ versus pre-immune $0.6\% \pm 1.1\%$, $P = 0.04$; paired two-tail t test) (Fig. 5B and D), suggesting that degradation of the cyst wall is necessary for the emergence of *G. lamblia* trophozoites.

To further elucidate the mechanism by which inhibition by

FIG. 3. Immunocytochemistry of cysts treated with anti-PCW serum in the presence or absence of sugars. (A) Preimmune serum did not bind to intact-appearing cysts. (B) Anti-PCW serum bound to intact cysts. Binding to nonintact cysts is also present, although some reactivity likely represents trapping of conjugate. (C) Preincubation of anti-PCW serum with chitotriose (100 mM) did not prevent binding to intact-appearing cysts. (D) Preincubation of anti-PCW serum with sialic acid (50 mM) prevented binding to intact-appearing cysts. Bar, 10 μ m.

FIG. 4. Immunoblot of reduced PCW antigens in the presence or absence of sugars. Lanes: 1, WGA-HRP; 2, WGA-HRP preincubated with chitotriose (100 mM); 3, WGA-HRP preincubated with sialic acid (50 mM); 4, anti-PCW serum; 5, anti-PCW serum preincubated with chitotriose (100 mM); 6, anti-PCW serum preincubated with sialic acid (50 mM). Sialic acid, but not chitotriose, eliminated recognition of PCW antigens by both WGA and anti-PCW serum. Sialic acid did not interfere with peroxidase or alkaline phosphatase activity.

FIG. 5. Appearance of cysts by differential interference contrast microscopy after excystation (stage III). Cysts appeared more degraded when they were preincubated in lectin solution (A) or preimmune serum (10%) (C) than when they were preincubated in WGA (10 μ g/ml) (B) or anti-PCW serum (10%) (D) suggesting that these ligands have interfered with the breakdown of the cyst wall during excystation. Ten times more of the cysts preincubated in anti-PCW serum retained the smooth, refractile, oval morphology of intact cysts than did cysts preincubated in preimmune serum. Solid arrows, intact cysts; open arrows, motile excysting trophozoites. Bar, 10 μ m.

these ligands occurs, the effect of timing of exposure to anti-PCW or WGA was examined. Inhibition of excystation by anti-PCW was most effective when cysts were incubated between stage I and stage II. Inhibition by WGA, however, was equally effective if cysts were incubated prior to stage I or between stage I and stage II (Fig. 6). Immunocytochemistry demonstrated that both anti-PCW and WGA bound during the preincubation prior to stage I were still present on the cyst surface after stage I (data not shown). WGA may bind more avidly and be more resistant to dissociation in the acidic and reducing stage I solution. WGA may also bind to nonprotein intrinsic cyst wall epitopes. Addition of anti-PCW serum or WGA after stage II had no effect on excystation.

Our results suggest that inhibition of excystation by anti-PCW serum or WGA is most likely mediated by interfering with a stimulus or process during stage II. This process may primarily involve degradation of the cyst wall, as increasing the concentration of trypsin during stage II partially reversed the inhibition by WGA and anti-PCW serum (Table 2).

DISCUSSION

The giardial cyst wall has previously been viewed primarily as an inert protective structure. Although biochemical analyses have yielded insights into cyst wall carbohydrate composition (11, 15) and demonstrated the presence of a variety of proteins (34) and glycoproteins (21, 25, 27), little is known about the interactions of the giardial cyst wall with the host milieu. Our ability to produce high-quality *G. lamblia* cysts in vitro allows us to avoid the problems associated with fecal cysts, particularly the unavailability of high-quality clinical specimens on a regular basis, difficulties in purifying sufficient cysts from stool

contaminants, biologic hazards of other enteric pathogens in clinical specimens, and extreme variation in biologic excystation efficiency (0 to >90%) (31). We demonstrate here that ligands that specifically bind to the wall of in vitro-derived cysts

FIG. 6. Effect of time of addition of anti-PCW or WGA on excystation. Percent excystation is compared with matched preimmune serum or buffer controls. Cysts were incubated in serum (10%) or WGA (10 μ g/ml) for 1 h at 4°C at the stage indicated. SI, stage I; SII, stage II.

TABLE 2. Partial reversal of inhibition of excystation by increasing trypsin concentration^a

Ligand	% Control excystation at trypsin concn (mg/ml)		
	1	5	10
WGA (10 µg/ml)	17.6 ± 6.5	64.3 ± 0.8 ^b	47.2 ± 5.0 ^b
Anti-PCW (10%)	6.7 ± 5.8	17.3 ± 9.3	38.6 ± 2.3 ^b

^a Cysts were preincubated for 1 h at 4°C with ligand or respective control and excysted in the usual manner, except for varying the trypsin concentration during stage II of excystation. Data are expressed as percent control excystation and are the means (± 1 SD) of two experiments.

^b $P < 0.05$, two-tailed paired t test, compared with 1-mg/ml trypsin.

efficiently inhibit excystation. Our results suggest that the inhibition may be mediated by binding to cyst wall carbohydrate epitopes, possibly in cyst wall glycoproteins.

Excystation is a complex process which occurs in response to relatively rapid and extreme changes in temperature and pH, as well as exposure to intestinal proteases. Upon ingestion by a new host, cysts pass from a cold, hypotonic environment with a pH of ~5 to 6 in water to the warm stomach with a pH of ~2 to 4, and then to the small intestine with a pH of ~7 to 8 (6). Bingham and Meyer first showed that exposure to acid was the key stimulus to excystation of fecal cysts in vitro (3). Subsequently, Rice and Schaefer demonstrated that the efficiency of excystation of fecal cysts in vitro was increased by exposure to a reducing environment and proteases (29), and we extended those findings to in vitro-derived cysts (4). There is little understanding, however, of the transduction of these external environmental signals across the cyst wall. Moreover, the physiologic cellular responses of mainly dormant cysts (22) to these changes have not been investigated previously.

We found that cyst cytoplasmic pH responded to changes in ambient pH, but that cytoplasmic pH was maintained closer to neutral than the external pH ($pH_{[out]}$). Anti-PCW serum did not appear to alter the overall change in $pH_{[in]}$ of cysts in response to changes in $pH_{[out]}$, indicating that inhibition of excystation by this ligand does not involve interference with the cytosolic pH response.

As excystation involves exposure to proteases, another possible mechanism for inhibition by anti-PCW serum and WGA is that bound ligand might prevent the digestion of an exposed protein(s) on the cyst surface that is required for signaling or for the emergence of the parasite. While there were clear changes in the pattern of proteins and glycoproteins recognized by anti-PCW serum and WGA after both stage I and stage II, we observed only subtle differences among cysts pretreated with anti-PCW serum, WGA, or controls in immunoblots after exposure to proteases. The surface-exposed portions of key cyst wall antigens that need to be cleaved or altered for excystation may be small. Therefore, inhibition of this alteration by bound antibody or WGA might not be detectable. Another possibility is that the (hypothetical) alteration is too subtle to be detected by immunoblots or that the targets are not extractable from the cysts under the conditions utilized for preparing immunoblots or reduced PCWs.

Our results support the hypothesis that both anti-PCW serum and WGA interfere with stage II stimuli, possibly by binding to sialic acid-containing glycoproteins, which may include the group II antigens. Free sialic acid may prevent the inhibition of excystation by occupying the binding site of the antibody, resulting in steric hindrance or conformational changes.

The most striking difference we observed between cysts pre-

treated with anti-PCW serum or WGA and the controls was the preservation of the morphologic appearance of many cysts after stage II. This suggests that the binding of anti-PCW or WGA prevented the normal breakdown of the *G. lamblia* cyst wall during excystation. Mechanisms by which bound ligand could interfere with excystation without affecting overall digestion of the antigens recognized by either ligand include inhibition of proteolysis, via steric hindrance, of adjacent cyst wall proteins; cross-linkage of cyst wall epitopes, preventing cyst wall conformational changes that allow release of emerging parasites; and interference with signaling across the cyst wall that may activate endogenous proteases or glycanases that digest the cyst wall. The ability of increased trypsin concentrations to partially reverse the inhibition supports a direct effect of bound ligand on cyst wall proteolysis; lack of complete restoration of excystation may be secondary to the inability of trypsin to remove the interfering ligand completely, preventing adequate access to the cyst wall surface.

Cyst wall antigens appear to be much more conserved (25) than the variable surface proteins that form the predominant surface antigen of trophozoites (2, 9, 19). Our original assumption had been that antibodies directed against cysts would not affect excystation because they would have to act on the external surface and because of the speed at which excystation occurs (<1 h). However, our present results suggest that cyst wall antigens might be protective immunogens, since blocking excystation could prevent infection.

Since as few as 10 viable cysts can establish infection (20, 28), inhibition of excystation would need to be complete or near complete in order to be effective. In addition, binding by endogenous mucosal antibodies would not occur until passage of the cysts into the upper small intestine and therefore would be competing with ongoing excystation. The presence of multiple proteases in the small intestine may also diminish the effectiveness of this inhibition. Although the relevant inhibitory antibody concentration present in our diluted anti-PCW serum (10%) is likely to be higher than any achievable concentration of mucosal antibodies in vivo, the "inoculum" is also much greater (1×10^6 to 5×10^6 cysts) than would be expected in a naturally acquired infection. Binding of anti-PCW antibody also appears to be relatively stable, as demonstrated by its continued inhibitory effect and its presence on the cyst surface after exposure to the reducing and acidic conditions of stage I solution. Vaccination of mothers may therefore be of benefit to breast-fed infants, as maternal secretory antibodies (17, 25) directed against the cyst wall might reduce the likelihood of acquiring infection. Finally, cysts coated with mucosal anti-cyst wall antibodies that are shed into drinking water supplies may be less infective, resulting in decreased community transmission in areas of poor sanitation.

These studies demonstrate the importance of cyst wall epitopes in excystation, provide the first insights into the physiologic responses of giardial cysts to specific environmental stimuli, and raise the possibility that antibodies directed against the cyst wall might interfere with the transmission of giardiasis. Further research is required to determine the exact mechanism(s) of inhibition.

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