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Identification and Localization of Cyst-Specific Antigens of *Giardia lamblia*

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We induced *Giardia lamblia* trophozoites to encyst in vitro by exposure to conditions which are specific to the human small intestinal milieu. We now show that encystation entails the appearance of two new groups of antigens detected in Western blots by rabbit antiserum against cysts which had been purified from human feces. A heterodisperse group of lower-molecular-mass antigens (~21 to 39 kilodaltons) was expressed relatively early (<19 h) in encystation. In contrast, discrete bands at ~66, 78, 92, and 103 kilodaltons only appeared after 24 h of incubation under conditions which lead to production of large numbers of water-resistant cysts. We also describe for the first time the appearance of prominent cytoplasmic vesicles, which were the earliest morphologic change in encysting trophozoites observable by light microscopy. Early in encystation, cyst wall antigens were concentrated in these vesicles, as shown by immunocytochemistry, suggesting that the vesicles function in export of cyst wall constituents.

Giardiasis is transmitted by ingestion of *Giardia lamblia* cysts from fecally contaminated water (4) or food (1). Little is known about the antigenic or biochemical composition of cysts because, until recently, they had not been produced in vitro. Because of our observation (11) that *G. lamblia* trophozoites encyst in the jejunum of experimentally infected (12) suckling mice, we proposed that specific small intestinal factors would promote encystation of cultured trophozoites in vitro (11).

In humans, *G. lamblia* trophozoites colonize the small intestine below the entry of the common bile duct (27). Therefore, in the lumen, they are exposed to biliary, pancreatic, and gastrointestinal secretions, which interact to form new physical structures. For example, bile salts form micelles with fatty acids cleaved from dietary lipid by pancreatic lipases (13). Moreover, the pH of the small intestine below the upper duodenum is rather high and may reach 7.6 to 8.0 (14). This slightly alkaline pH increases ionization of fatty acids, promotes their association with bile salts (13), and decreases their toxicity to *G. lamblia* in vitro (18).

We have shown that exposure of cultured *G. lamblia* trophozoites to small intestinal factors (primary bile salts and fatty acids [11]) at pH 7.8 leads to induction of large numbers of water-resistant cysts (10), some of which are capable of excystation and infection of suckling mice (F. D. Gillin, *in* P. M. Wallis and B. R. Hammond, ed., *Advances in* Giardia *Research*, in press).

These studies contain an analysis of the expression of cyst-specific antigens which are recognized by rabbit antiserum against *G. lamblia* cysts purified (6) from human feces (11). We had observed cyst antigens by indirect immunofluorescence early in encystation, prior to morphologic differentiation, as well as in mature cysts (11). By morphologic differentiation, we mean the microscopically observable, gradual transformation of the motile, half-pear-shaped trophozoite into the oval, refractile cyst form (see reference 8 for description of both stages). Therefore, we have now used Western blotting to analyze the expression of individual cyst antigens during encystation. We show that the synthesis of cyst antigens is not coordinately regulated because a heterodisperse group of lower-molecular-mass antigens (~21 to 39 kilodaltons [kDa]) appear early in encystation, while four discrete bands (~66 to 103 kDa) appear later and only under conditions (10) which lead to production of large numbers of water-resistant cysts.

We also report for the first time the appearance of prominent cytoplasmic vesicles containing cyst antigens in trophozoites which are undergoing encystation. Reactivity with cyst-specific antiserum is localized in these vesicles and the nascent cyst wall, supporting the idea that these vesicles function in export of cyst wall antigens.

MATERIALS AND METHODS

Trophozoite cultivation and induction of encystation. *G. lamblia* WB (ATCC 30957) trophozoites were grown in plastic tissue culture flasks (25 cm^2; Corning) to late log phase in TYI-S-33 medium (5) with 0.5 mg of bovine bile per ml (15) and 10% bovine serum at pH 7.0 to 7.1. Unattached trophozoites were decanted, and the remaining monolayer of attached cells was refed with freshly prepared encystation medium.

Encystation medium. To filter-sterilized TYI-S-33 medium, modified by omitting the bovine bile and adjusting the pH to 7.8 with 1 M NaOH, was added a 20-fold-concentrated lipid supplement containing sodium glycocholate (GC), myristic acid (MA), and oleic acid (OA; sodium salt). The lipid dispersion was prepared by adding enough solid GC, MA, and OA to boiling double-distilled water so that the final concentrations of these lipids were 10, 0.5, and 0.1 mM, respectively, in the encystation medium. The hot lipid supplement was vortexed and immediately added to the modified TYI-S-33 medium, as were the antibiotics piperacillin (500 μg/ml; Lederle Laboratories) and amikacin (125 μg/ml; Bristol Laboratories), which do not affect *G. lamblia* growth (9) or differentiation (11), but were required because the bile salt-fatty acid dispersion was not filter sterilized. Unless otherwise indicated, chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) and were of the highest

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quality available. Variations from this standard protocol are indicated in each figure legend.

Antigen preparation. Unless otherwise specified, encysting cultures were incubated for 48 h at 37°C, since the greatest number of water-resistant cysts could be recovered at this time. To detect all antigen expression, total cultures were harvested by chilling the flasks for 20 min at 0 to 4°C and mixing. Cells were then centrifuged for 10 min at 833 × *g*, and the pellet was immediately resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (4% SDS, 0.125 M Tris, 20% glycerol, 100 mM dithiothreitol, 0.007% bromphenol blue) at a concentration of 3×10^8 cells per ml, heated for 6 min at 100°C, and frozen at −70°C until use.

SDS-PAGE and Western blot analysis. SDS-PAGE was performed by the method of Laemmli (16) with a 3% acrylamide stacking gel and an 8.5% acrylamide running gel. A total of 20 μl of cyst antigen was applied to each lane of the gel. The electrophoretically separated proteins were transferred to nitrocellulose paper (0.2 μm pore size) according to the method of Towbin et al. (24) by transferring them overnight at 30 V and then for 2 h at 70 V. The nitrocellulose membrane was then soaked for 2 h in a 0.3% gelatin–0.005% Tween-20–phosphate-buffered saline (PBS) (pH 7.2) solution, which was also used for all subsequent incubations and washes. Cyst antigens were visualized by reacting with rabbit anticyst or preimmune serum (1:200) for 2 h at room temperature and then for 1 h in protein A-peroxidase conjugate (1:2,000; Zymed Laboratories) and developed in 4-chloro-1-naphthol substrate. The rabbit anticyst serum was prepared as described before (11) by intravenous injection of cysts which had been purified extensively (6) from the feces of a patient with chronic, symptomatic disease. This method of immunization was selected to minimize breakage of cysts and release of trophozoite antigens. The reaction with cysts from axenic cultures shows that the antigens are specific to the parasite and are not of fecal origin. Moreover, since the antiserum was prepared against fecal cysts, the antigens cannot be derived from the culture medium. Molecular masses were calculated with reference to prestained markers included in each gel.

Immunocytochemical localization of cyst antigens. Encysting or control parasites were harvested by chilling and centrifuging. The cells were resuspended in one-fifth volume of PBS, and 40 μl was spotted within an indelible-ink circle drawn on a glass slide. After attachment for 10 min at 37°C in a humidified chamber, 40 μl of warm 6% (vol/vol) glutaraldehyde was used to fix the attached cells for 2 min and then removed by vacuum aspiration. The fixed cells were rinsed three times with PBS and then permeabilized by quickly dipping the slides twice in cold methanol (−20°C) and rinsing them immediately three times with PBS. Slides were then flooded with rabbit anticyst or preimmune serum (diluted 1:50 in PBS containing 0.05% Tween-20 and bovine serum albumin [10 mg/ml]) and incubated for 1 h at 37°C in a humidified chamber. After being rinsed thoroughly with PBS, the slides were flooded with protein A-peroxidase (1:1,000 in the same PBS–Tween-20–bovine serum albumin buffer) and incubated for 1 h at 37°C in a humidified chamber. Slides were then rinsed three times with PBS and developed with 4-chloro-1-naphthol substrate. Cells were mounted in H_2O, viewed with a Nikon Optiphot microscope with Nomarski differential interference contrast optics, and photographed with Technical Pan film.

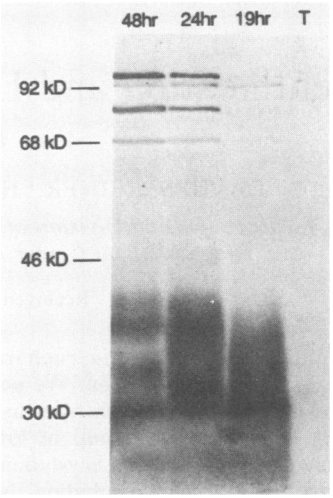

FIG. 1. Immunoblot of cultures encysting for 19, 24, or 48 h and control, nonencysting trophozoites (T) with rabbit antiserum raised against purified fecal cysts. The positions of prestained molecular mass markers are shown to the left.

RESULTS

Analysis of cyst antigens in immunoblots. Since earlier studies (11) had shown that cyst antigens began to appear prior to morphologic differentiation and development of water resistance, we asked when these antigens are expressed and whether they are all expressed in parallel. Therefore, total parasite cultures were harvested after increasing times in encystation medium, and samples containing equal numbers of parasites were subjected to SDS-PAGE and Western blotting with our rabbit anticyst serum (Fig. 1).

As we reported earlier with different methods (11), this serum did not react detectably with trophozoite antigens from control nonencysting cultures. Maximum cyst antigen expression was observed at 24 to 48 h, whereas water resistance develops at ~40 to 48 h (data not shown). The major antigens consisted of a polydisperse complex at ~21 to 39 kDa and discrete bands at ~66, 78, 92, and 103 kDa. The pattern of antigens in the total culture at 48 h was the same as that of water-resistant cysts harvested at the same time (not shown). Each cyst contains a doubled trophozoite; therefore, solubilization in SDS-PAGE sample buffer released both the trophozoite and cyst antigens. The latter were present only in lanes containing encysting cells, whereas trophozoite antigens were present in all lanes but not visualized by the cyst-specific serum. The presence of trophozoite antigens was demonstrated by staining for protein or reactivity with antiserum against trophozoites (not shown).

The cyst antigens were not all synthesized synchronously, since the lower-molecular-mass antigens appeared earliest. The 21- to 39-kDa antigens began to appear as early as ~4 h and increased gradually until ~24 h (not shown), while the 66- to 103-kDa antigens began to appear between 19 and 24 h in encystation medium (Fig. 1).

We next examined the effects of pH and bile salt-fatty acid inclusion on expression of cyst antigens (Fig. 2). Parasites were incubated for 48 h at pH 7.0, 7.4, or 7.8 in the absence or presence of GC, MA, and OA. Significant numbers of water-resistant cysts were produced only at pH 7.4 or 7.8 in the presence of GC, MA, and OA (10). Similarly, the 66- to 103-kDa antigens were efficiently expressed only under these

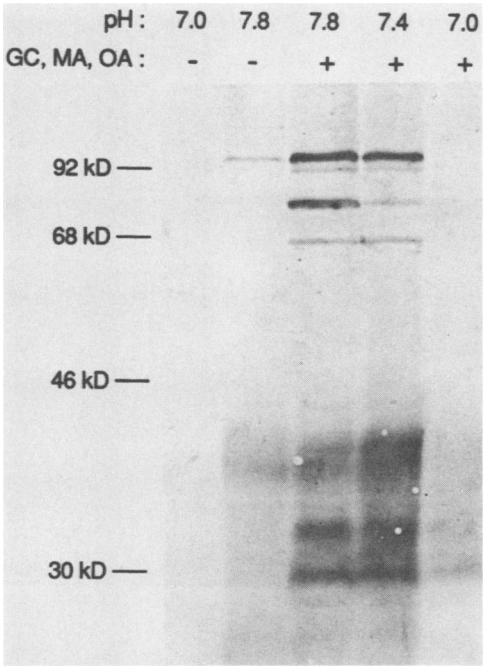

FIG. 2. Immunoblot analysis of the effects of pH and encystation stimuli on expression of cyst antigens at 48 h.

conditions. In contrast, the 21- to 39-kDa antigens were partially expressed at pH 7.8 in the absence of GC, MA, and OA and at pH 7.0 in their presence. Our initial encystation experiments were carried out at pH 7.0, and we observed only the 21- to 39-kDa antigens (11). Thus, these antigens appear to be less stringently controlled. No cyst antigens were observed at pH 7.0 without bile salt or fatty acids (Fig. 2).

In previous experiments (10), we found that many fatty acids could stimulate encystation. Therefore, we examined the effects of substituting fatty acids on cyst antigen expression. We observed identical cyst antigen patterns after incubation with myristic acid (C_{14}), pentadecanoic (C_{15}), and palmitic acid (C_{16}) at pH 7.8 in the presence of OA and sodium GC (data not shown). We have not yet studied antigen patterns with GC and OA alone, since fewer water-resistant cysts were observed (10).

As an initial step in characterization, antigens prepared under standard encystation conditions were separated by SDS-PAGE, transferred to nitrocellulose, and treated with trypsin (150 μg/ml) in the presence or absence of excess soybean trypsin inhibitor (500 μg/ml). After being blocked and washed with gelatin-containing buffer, the strips were reacted with anticyst serum or peroxidase-conjugated wheat germ agglutinin (WGA). All reaction with the antibody was ablated by treatment with trypsin alone. In contrast, the reactivity with WGA was not removed (not shown). Thus, these antigens are proteinaceous in composition.

Location of cyst antigens in specific vesicles. During examination of live cells early in encystation, we observed discrete vesiclelike structures which appeared to be protruberances by differential interference contrast (DIC) microscopy (Fig. 3) or dark granules by phase contrast microscopy (not shown). However, few if any such vesicles have been observed in nonencysting trophozoites (8). Since the encystation-specific vesicles (ESV) appeared in parallel with cyst antigen expression in immunoblots, we used immunocytochemistry to determine whether these antigens are localized within the vesicles.

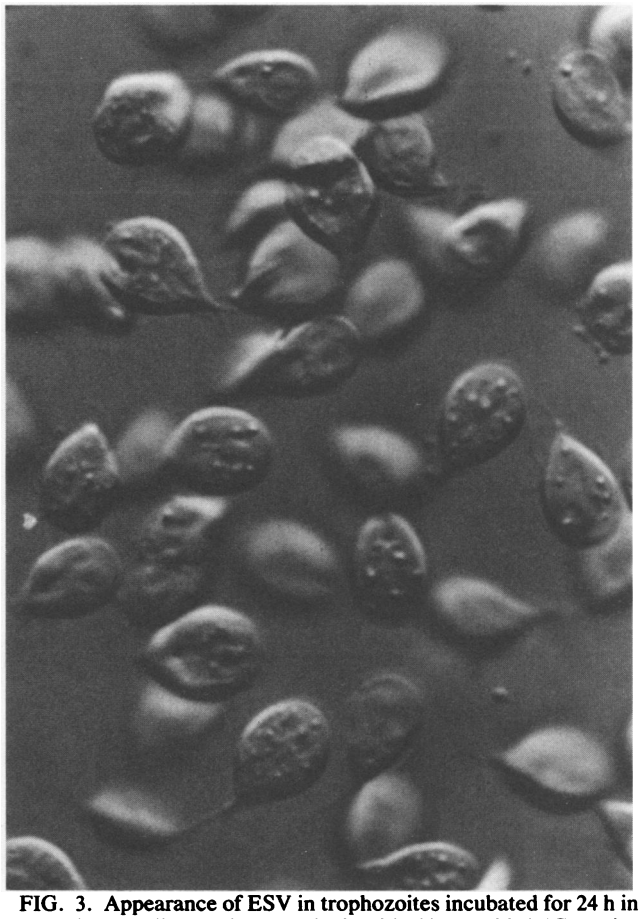

FIG. 3. Appearance of ESV in trophozoites incubated for 24 h in encystation medium, photographed with Nomarski DIC optics (×1,300).

In preliminary experiments, the morphology of cells fixed with glutaraldehyde (Fig. 3) was the same as that of live cells (not shown) by DIC optics. Brief permeabilization of fixed cells was necessary for antibody reaction with the ESV and did not greatly disrupt cellular morphology. Early in encystation (~18 to 24 h), reactivity with anticyst serum was localized in the ESV and at the periphery of some cells (Fig. 4 and 5). Later in encystation, entire cysts stained black (Fig. 6). Control preimmune rabbit serum was not reactive (Fig. 7). Moreover, nonencysting cells lacked ESV and did not react with anticyst serum. Thus, the cyst-specific antigens are concentrated in the ESV and, to a lesser extent, the cell surface prior to morphologic differentiation.

DISCUSSION

These studies are the first detailed characterization of cyst-specific antigens of *G. lamblia*. Since these antigens are present both during encystation and in mature, water-resistant cysts derived in vitro from axenic cultures, they are specific to *G. lamblia* and not of fecal origin. Furthermore, their expression is developmentally regulated during encystation. A prominent disperse group of lower-molecular-mass antigens (~21 to 39 kDa) appears early (~4 h) in encystation, while four discrete bands of ~66 to 103 kDa appear only after ~24 h. The later, higher-molecular-mass antigens are

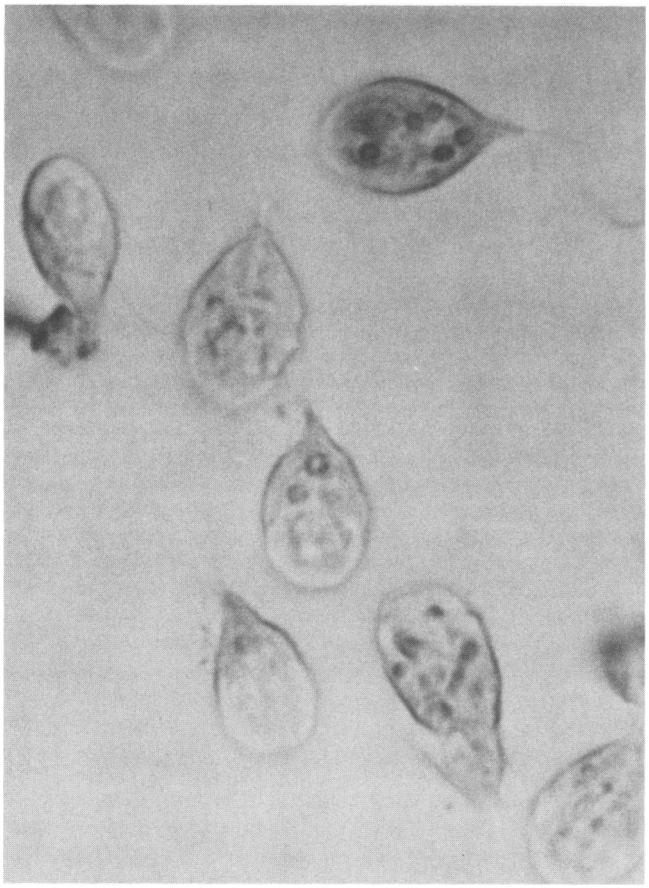

FIG. 4. Immunoperoxidase localization of cyst-specific antigens in fixed, permeabilized cells which had been incubated for 18 h in encystation medium. Reaction with rabbit anticyst serum is observed in specific vesicles and at the cell periphery (×1,960).

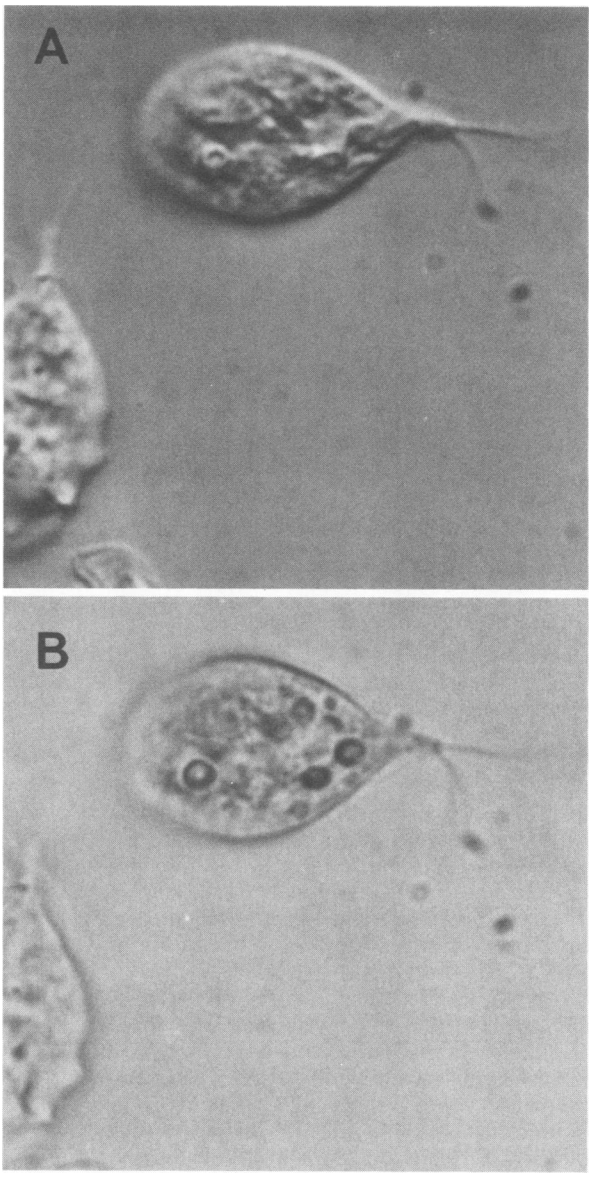

FIG. 5. Higher magnification micrographs of a cell shown in Fig. 4, photographed (A) with DIC optics to show the vesicles and (B) showing the immunoperoxidase reaction product in vesicles and at the sides of the cell (×2,975).

expressed only under conditions which lead to production of large numbers (~10^5/ml) of water-resistant cysts (10) at 42 to 48 h. Thus, these two groups of antigens appear to be under independent control, but both are expressed before morphologic differentiation and development of an intact cyst wall. Under the conditions used, they are not detected in control, nonencysting trophozoites by indirect immunofluorescence (11), immunocytochemistry, or Western blots, suggesting that they are not present in cryptic or precursor form.

Sauch (21) first prepared antibodies against purified fecal *G. lamblia* cysts which are stage-specific and useful for detecting cysts in water supplies. This antiserum reacts with viable, in vitro-derived *G. duodenalis*-type cysts from the MR4 muskrat isolate (22). The corresponding antigens have not been characterized. Rosoff and Stibbs (19, 20) raised monospecific antibodies against a 65-kDa highly glycosylated antigen which is present in the cyst wall. Since this antigen was also reported in trophozoites and is trypsin resistant (20), it probably does not correspond to the cyst antigen of ~66 kDA which we observed.

The antigens we observed were conserved between two isolates of widely differing geographic origin, since the antiserum was raised against cysts from the feces of a San Diego patient with no history of travel, while the in vitro cysts were derived from strain WB, which had been isolated from a patient infected in Afghanistan (23). In related recent studies, we observed that many human sera react with some or all of the ~66- to 103-kDa cyst-specific antigens (unpublished), but rarely with the 21- to 39-kDA antigens. We are currently determining whether this reactivity is related to infection and examining the possibility that these antigens contain epitopes in common with other microbes.

Our rabbit anticyst serum reacts with unfixed fecal cysts by IFA (11) and with the wall of both in vitro-prepared and fecal cysts by immunoelectron microscopy (unpublished). Therefore, some if not all of the antigens are in the cyst wall. The presence of chitin was reported earlier in *Giardia* cyst walls (25), and we have observed increased activity of chitin synthetase in extracts of encysting parasites (S. Das and F. D. Gillin, Abstr. Annu. Meet. Am. Soc. Biol. Chem. 1987, abstr. 1295).

To begin to characterize the antigens recognized by our rabbit anticyst serum, we investigated the possibility that

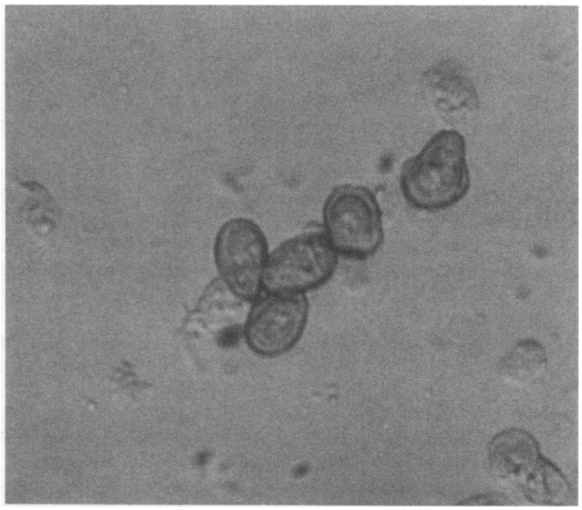

FIG. 6. Immunoperoxidase localization of cyst-specific antigens in fixed, permeabilized cells which had been incubated for 2 days in encystation medium, showing reaction product concentrated in the cyst walls (×1,050).

they are glycosylated. Ward et al. (26) reported that certain trophozoite surface antigens react with WGA. The four cyst antigens of 66 to 103 kDa may contain exposed *N*-acetylglucosamine or sialic acid since they react with WGA-peroxidase on nitrocellulose blots. Of the WGA-reactive bands in an adjacent lane containing only trophozoite antigens, only a faint band at 78 kDa was similar in molecular mass to a cyst antigen. Therefore, with this possible exception, the WGA-reactive cyst antigens do not appear to correspond to those in trophozoites.

The idea that the major epitopes recognized by our anticyst serum are protein rather than carbohydrate is supported by our observation that trypsin treatment of these antigens on nitrocellulose blots ablated all subsequent reactivity with this antiserum. In contrast, the reactivity with WGA remained, suggesting that the carbohydrate moieties were not removed by the trypsin treatment. Furthermore, concanavalin A-reactive determinants, which also appear during encystation, were not recognized by our rabbit anticyst serum (not shown). At present, we do not know whether the cyst antigens are newly synthesized or whether they contain some modified trophozoite determinants which are not recognized by our anticyst serum. Nonetheless, it is clear that the appearance of these antigens is an encystation-specific event.

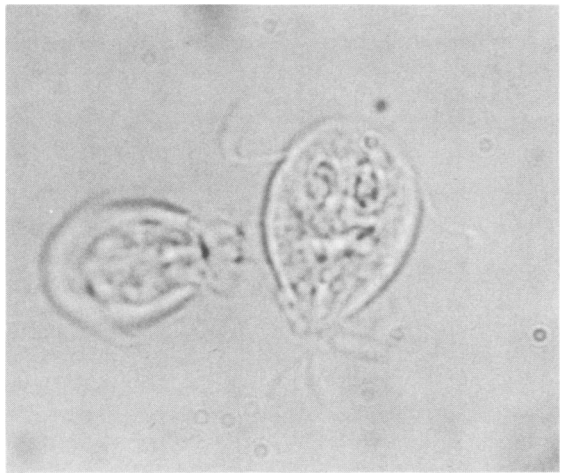

FIG. 7. Control immunoperoxidase reaction of fixed, permeabilized cells which had been incubated for 18 h in encystation medium. There was no reaction with preimmune serum (1:50) from the same rabbit as the anticyst serum (×2,500).

The prominent vesicles we have described here are the earliest morphologic feature of encystation visible by light microscopy. They began to appear at ~4 h in encystation medium, before cells began to round up and detach, but were rare in control, nonencysting trophozoites. These ESV are not the same as the array of 100- to 400-nm membrane-bound, electron-translucent peripheral vesicles which can be visualized by transmission electron microscopy (8). The latter underlie the cell membrane (8), can take up exogenous ferritin (3), and have alkaline phosphatase activity (7). Therefore, they may correspond to lysosomes (17). We do not yet know whether there is any structural or functional relationship between ESV and the peripheral vesicles.

The ESV appear as protruberances with Nomarski DIC optics or as dark granules under phase contrast (not shown). It is likely that the appearance under DIC is a reflection of physical properties of the ESV, e.g., optical density differences integrated through the depth of the cell rather than surface topography. This is supported by transmission electron microscopy, which shows that ESV are ~1.6 to 2.6 μm in diameter, membrane-bound, electron-dense vesicles, distributed in the cytoplasm. Some of the ESV are just beneath and protrude from the plasma membrane (unpublished).

We have shown by enzyme immunocytochemistry that the cyst antigens are localized in ESV early in encystation, whereas later the entire cyst is reactive. Immunoelectron microscopy confirms that the cyst antigens are concentrated within the ESV and also in the developing cyst wall. Moreover, membranes of protruding ESV containing immunogold label have been observed to be contiguous with the plasma membrane, suggesting that they can release the vesicle contents into the nascent cyst wall (unpublished). These results support the idea that ESV function in export of cyst antigens for assembly of the wall.

Thus, encystation, a complex differentiation process, entails the regulated synthesis of stage-specific antigens. Studies are under way to determine the nature of these antigens and the mechanism of regulation.

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