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Appearance of Sialoglycoproteins in Encysting Cells of *Entamoeba histolytica*

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Amoeba-bacterium cultures of *Entamoeba histolytica* transferred to a hypoosmotic medium depleted of nutrients changed morphologically and biochemically. The cells ejected grains of rice starch, rounded up, and formed a distinct cell wall that was resistant to detergent, bound the sialic acid-specific lectin from *Limulus polyphemus*, and became fluorescent with Calcofluor M2R. A subpopulation of these cells displayed more than one nucleus. All these signs are characteristic of encysting cells and were also observed in cysts obtained from a human patient. The morphological changes were accompanied by the appearance of two new glycoproteins with apparent molecular sizes of 100 and 150 kilodaltons which contained sialic acid. Sialic acid has been reported to be absent from trophozoites of *Entamoeba* species. The presence of this sugar residue on cyst-specific proteins parallels recently reported findings during the encystation of the related reptilian parasite *Entamoeba invadens*. This may indicate a basic role for sialic acid in the encystation of *Entamoeba* parasites.

Cysts of *Entamoeba histolytica* are the infective stage of the parasite and the form that is most commonly seen during examination of human stools (19). Biochemical studies on this stage of the parasite have been hampered by lack of material since no in vitro culture system exists that can produce good and reproducible yields of cysts. No cyst formation of *E. histolytica* has ever been reported in axenic culture. Xenic cultures of individual strains of *E. histolytica* that produced cysts in response to addition or deprivation of nutrients (6, 8, 15, 36; D. Mirelman and B. Avron, *in* J. I. Ravdin, ed., *Amebiasis: Human Infection by Entamoeba histolytica*, in press) or changes in redox potential (9, 10) have been reported. These methods varied widely and depended on particular strains of *E. histolytica* which are not available now. The only common factors appear to be an appreciation of a particulate source of carbohydrate and the nature, though undetermined, of the bacterial flora in the cultures.

Sialic acids on the surface of cells have been shown to be important in both multicellular organisms (30, 33) and protozoal parasites (32). Although sialic acid has been reported to be absent from trophozoites of *Entamoeba* species (16, 23), recent work has demonstrated the appearance of stage-specific sialylated glycoproteins during the encystation of the reptilian parasite *E. invadens* (3a) Stolarsky, R. Schauer, G. Reuter, and D. Mirelman, Mol. Biochem. Parasitol., in press). This parasite produces cysts in culture in response to hypoosmotic shock and the depletion of nutrients (5, 28). In the present study, we determined the effect of a hypoosmotic medium on xenic cultures of *E. histolytica*. Under these conditions, the cells rounded up, became resistant to detergent, and showed the appearance of two new sialylated glycoproteins.

MATERIALS AND METHODS

Abbreviations. FITC, Fluorescein isothiocyanate; kDa, kilodaltons; LPA, *Limulus polyphemus* agglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; WGA, wheat germ agglutinin.

Cultivation of trophozoites and preparation of encysting cells. Trophozoites of *E. histolytica* isolate WI:0385:191, obtained by P. G. Sargeaunt in March 1985 from an asymptomatic carrier in Israel, were cultivated in 15-ml glass tubes containing 11 ml of either Diamond TYSGM-9 medium (14) or Jones monophasic medium (18) modified as described previously (25). For the encystation experiments, cells grown in the Jones medium for 48 h were harvested by chilling the tubes and sedimenting the cells (300 × *g*, 5 min). The cells were suspended at a concentration of 10^5/ml in a solution of $MgSO_4 \cdot 7H_2O$ (65 g/liter) containing 5% ox serum and an antibiotic mixture composed of erythromycin (Sigma Chemical Co., St. Louis, Mo.), amikacin (Bristol Laboratories, Syracuse, N.Y.), and cefotaxime (Hoechst, Frankfurt, Federal Republic of Germany), each at 50 μg/ml final concentration. The osmotic pressure of the Jones growth medium as measured with an osmometer (Osmotic Precision, Sudbury, Mass.) was 400 mosmol/kg. The osmotic pressure of the magnesium sulfate-serum solution was 300 mosmol/kg.

After 48 to 72 h in the magnesium sulfate medium, the cells were harvested by cooling the culture bottles and sedimenting the cells (300 × *g*, 5 min). The resistance to detergent was measured by washing the cells in water and then suspending them in a solution of 1% Vagisec (Schmid Products) and vigorously vortexing the suspension. The yield of detergent-resistant cells was routinely approximately 75% of the initial number of trophozoites. These cells were over 50% viable as detected by exclusion of eosin and gave rise to viable cultures of trophozoites when reinoculated into Jones culture medium. Trophozoites were all lysed by the detergent conditions used.

Staining of cysts. Cells were stained with malachite green (BDH, Poole, England) to detect the chromatoid bodies by suspending the cells at a concentration of 10^6/ml in saline containing 10% formaldehyde for 20 min, followed by the addition of 0.33 volume of diethyl ether and sedimentation (1,000 × *g*, 5 min). The pellet of cells was mixed with an equal volume of a solution of 1% acetic acid containing 0.1%

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malachite green. Cells were also stained with Lugol iodine stain (24) and 0.1% Calcofluor M2R (3).

Staining of cells with fluorescent lectins. Cells were harvested and stained with FITC-coupled WGA (Bio-Yeda, Nes Ziona, Israel) or FITC-conjugated lectin from *Limulus polyphemus* (FITC-LPA; EY Laboratories, Inc., San Mateo, Calif.) used at a concentration of 100 μg/ml. The method was as described previously (3a). Sialic acid inhibition of staining was assessed by preincubating the lectin for 20 min with 0.2 M sialic acid. The sugar was also present during the staining.

Preparation of cysts from human material. Human feces (4 to 5 g) from an asymptomatic carrier of *E. histolytica* were suspended in saline and filtered through gauze. An enriched population of cysts was obtained by repeated centrifugation (600 × *g*, 10 min) over a cushion of 0.85 M sucrose. The cysts were harvested from the interface.

Metabolic labeling. Trophozoites at logarithmic growth (10^6) in the TYSGM-9 medium were chilled to 4°C, harvested by centrifugation (5 min, 300 × *g*), and transferred to new growth medium (11 ml) including 20 μl of [^{35}S]methionine (1,130 Ci/mmol, 15.6 mCi/ml in aqueous potassium acetate [20 mM] containing β-mercaptoethanol [0.1%]; Radiochemical Centre, Amersham, England) or D-[1,6-^{3}H]-*N*-glucosamine hydrochloride (20 μl, 42.4 Ci/mmol, 1 mCi/ml; New England Nuclear Corp., Boston, Mass.). The cells were then incubated at 37°C for 48 h, harvested as described above, and washed (three times for 5 min, 300 × *g*) with 20 mM phosphate-buffered saline, pH 7.2.

To label the encysting cells, trophozoites (4×10^6) were harvested from Jones medium in the logarithmic growth phase and centrifuged (300 × *g*, 5 min). The cell pellet was suspended in the hypoosmotic magnesium sulfate medium (40 ml) containing 5% bovine serum and 80 μl of [^{35}S]methionine or D-[1,6-^{3}H]-*N*-glucosamine hydrochloride (80 μl; both of the same specifications as above). The cells were incubated at 37°C and harvested after 72 h as described above.

Chitinase treatment of encysting cells. Cells metabolically labeled with [^{3}H]glucosamine (as above) were treated with Vagisec (1%) followed by an incubation with 1 M NaOH (2 h at 100°C). The NaOH was neutralized with an equivalent volume of HCl (1 M), and the solution was dialyzed extensively against water. The nondialyzable material was divided into two portions and then lyophilized, and each portion was suspended in 2 ml of 20 mM MES (morpholineethanesulfonic acid) buffer (pH 6.5). One aliquot was incubated overnight at 37°C with chitinase (500 μg/ml; CalBiochem-Behring, La Jolla, Calif.), while the other was similarly incubated but without enzyme. The digests were lyophilized, dissolved in water (100 μl), applied to paper chromatograms (Whatman no. 3), and run for 20 h in *n*-butanol–acetic acid–water (4:1:5, vol/vol/vol). After the run, the paper was allowed to dry, strips (1 cm wide) were cut, and the radioactivity was determined in a scintillation counter. Markers for the migration of *N*-acetylglucosamine oligosaccharides were prepared from partial acid hydrolysates of chitin (Sigma) and detected on the paper chromatograms as previously described (26).

Labeling of cells and glycoproteins with NaB[^{3}H]$_4$. The method used to label cells and glycoproteins was as described by Gahmberg and Anderson (17). *E. histolytica* cells (1×10^6 to 2×10^6) were treated with 2 mM sodium periodate (10 min) at 4°C, washed with phosphate-buffered saline (300 × *g*, 5 min), and reduced with NaB[^{3}H]$_4$ (15 μl, 452 mCi/mmol; Radiochemical Centre) for 30 min. Controls which were not pretreated with sodium periodate were also reduced with NaB[^{3}H]$_4$. After the labeling, the cells were washed as before and solubilized in boiling sample buffer for SDS-PAGE as described below.

Glycoproteins (20 μg/ml) were labeled as cells except that after reduction with borohydride, the protein was precipitated in 10% trichloroacetic acid and sedimented (20,000 × *g*, 15 min). The precipitate was washed four times with cold 5% trichloroacetic acid and dissolved in water.

Detergent and salt treatment of cells. Cells labeled with NaB[^{3}H]$_4$ were suspended in 10 mM Tris buffer (pH 8) and subjected to treatment with 0.5 M NaCl, nonionic detergent (0.5% Nonidet P-40; Sigma), or ionic detergent (1% sodium deoxycholate; Sigma) for 15 min at 4°C and then centrifuged (300 × *g* for 5 min). The pellet and supernatant were counted to determine the percentage of radiolabel that was solubilized. The cell pellet was suspended in sample buffer for gel analysis (see below) and compared with the supernatant to see whether the radiolabeled proteins could be removed from the cells by these treatments.

SDS-PAGE. Cells were harvested as described above, washed three times with 0.9% NaCl (saline, 300 × *g*, 5 min), and suspended at a concentration of 10^7 cells per ml in a boiling sample buffer consisting of 0.18 M Tris (pH 6.8), 9% SDS, 40% sucrose, 2 M β-mercaptoethanol, and bromophenol blue (2 μg/ml). The samples were boiled for a further 5 min in a water bath. Electrophoresis was routinely done on 7.5 to 15% gradient acrylamide gels (20). The material loaded per gel lane was equivalent to 3×10^5 amoebal cells.

Proteins were transferred from SDS-polyacrylamide gels to nitrocellulose filters as previously described (12).

Controls were done in which only the bacteria which were associated with the cultures of amoebal cells were prepared and subjected to the same SDS-PAGE analysis to detect any contamination by bacterial components. The supernatant obtained when amoebal cultures were sedimented (300 × *g*, 5 min) and harvested contained almost all the bacteria that were associated with the amoebae (6×10^8 bacterial cells per ml). This supernatant was collected and recentrifuged (10,000 × *g*, 10 min) to sediment the bacteria, which were then resuspended in a volume of sample buffer equal to that of the amoebal cells from the same culture (6×10^{10} bacterial cells per ml). As a control for possible protease degradation in trophozoites compared with cysts, a sample of trophozoites and encysting cells were combined during the harvesting and processed together for SDS-PAGE analysis. In an experiment to assess the contribution and presence of glycoproteins from serum to the encysting cells, the gel profile of the total amount of serum available in the encystation medium was compared with that of the encysting cells (6 μl of serum and 6×10^4 encysting cells).

Proteins were stained on gels with Coomassie stain (0.1% Coomassie brilliant blue R250 [Sigma] in 42% methanol with 10% acetic acid) for 30 min and destained overnight in a solution of 20% methanol and 6% acetic acid. Carbohydrates were stained on the gels with Schiff reagent as described previously (12, 37).

Gels containing radiolabeled samples were dried under vacuum and exposed to X-ray film.

TLC. Tritiated proteins run on SDS-polyacrylamide gels and blotted on nitrocellulose filters were identified by staining with Ponceau S (0.1% in 7% trichloroacetic acid; Sigma) for 1 min, followed by destaining in water. The identified bands were excised, and the tritiated sugars were released by hydrolysis in 0.05 M H_2SO_4 for 1 h at 80°C (35). The filter was removed, and the remaining solution was neutralized with an equivalent amount of barium chloride. This gave rise to a precipitate of barium sulfate which was sedimented in an

Eppendorf centrifuge (10,000 × *g*, 10 min). The supernatant was lyophilized, and then the sample was redissolved in water and loaded onto 0.2-mm thin silica gel plates. Chromatography was performed in a solvent system of *n*-propanol–water (7:3, vol/vol) (31). At the end of the run, the plates were dried and the silica was scraped off in regions 0.5 cm from the origin, added to scintillation fluid, and counted.

Electroelution of 150-kDa protein. Preparations of the encysting cells were run on SDS-polyacrylamide gels; and the area of the gel that included the 150-kDa protein, as identified by parallel tracks that were stained with Coomassie blue, was cut out and inserted into a dialysis bag, and the protein was electroeluted out of the gel in a buffer consisting of 0.2 M glycine, 25 mM Tris, and 0.1% SDS. Electroelution was at 4°C overnight at a constant current of 20 mA. The material was removed from the gel and dialyzed against three changes of methanol followed by three changes of water and then lyophilized.

Removal of sialic acid. Proteins from cells labeled with $NaB[^3H]_4$ (see above) were identified on a nitrocellulose blot as described for the TLC. The piece of filter that included the protein was excised and incubated for 16 h at 37°C in 40 mM sodium acetate (pH 5) (500 μl) containing 30 mU of sialidase from *Vibrio cholerae* (Boehringer GmbH, Mannheim, Federal Republic of Germany). Controls without enzyme were incubated in parallel. After the incubation, the supernatant and filter were counted, separately, in a scintillation counter.

In another type of experiment to identify the material that was digested by the enzyme, nonradioactive protein preparations from cells were run on SDS-polyacrylamide gels and the 150-kDa area of the nitrocellulose blot (see above) was excised and labeled with $NaB[^3H]_4$ as described above for cells. The piece of filter was then incubated with sialidase as already described. The supernatant was lyophilized and analyzed on silica gel TLC plates as described above.

Asialofetuin was prepared basically as described by Spiro (34). Briefly, fetuin (100 mg) that had been radiolabeled with $NaB[^3H]_4$ (as above) was incubated for 1 h at 80°C in 0.05 M H_2SO_4 (400 ml). The solution was then dialyzed extensively (four times) against water and lyophilized.

Carbohydrate analysis by gas chromatography. Preparation of sample and analysis by gas chromatography were as described previously (13). Briefly, an internal standard of 50 nmol of mannitol and 100 nmol of perseitol was added to sample. The sample was hydrolyzed by methanolysis and run on a 3% SE-30 Gas-Chrom Q column (100/120 mesh), using a Packard gas chromatography system preprogrammed for a temperature increase of 2°C/min. Separation was performed between 130 and 210°C, and sialic acid was retained by the column up to a temperature of 200°C, emerging after 35 min.

RESULTS

When trophozoites of *E. histolytica* WI:0385:191 were transferred from the modified Jones (18) growth medium to the solution containing magnesium sulfate and serum, the cells ejected the ingested rice starch particles, rounded up, and formed cell clumps which dispersed over the following 48 h. The rounded cells remained viable (>50%) as determined by exclusion of 1% eosin. After 48 h in the hypoosmotic medium, the cells (>75%) were resistant to detergent (1% Vagisec), in contrast to the trophozoites from Jones medium, which were disrupted (none visible, less than 1% remaining). Microscopic observation of the rounded cells stained with Lugol iodine or malachite green (Fig. 1) re-

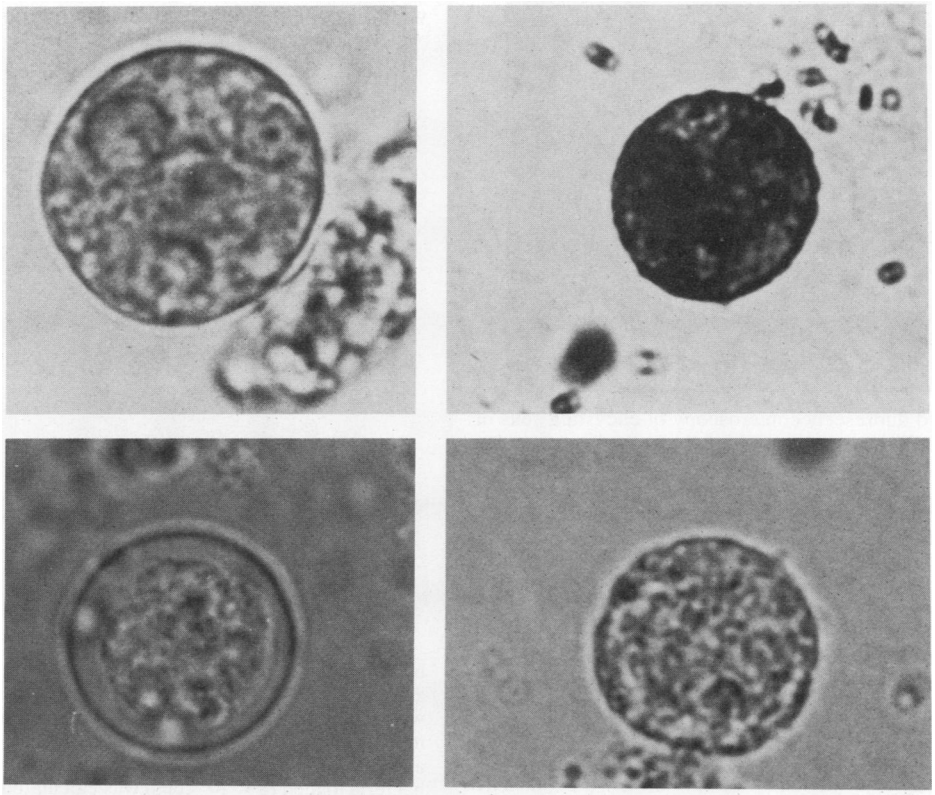

FIG. 1. Encysting cells of *E. histolytica* WI:0385:191 in culture (×1,000). Top right cell stained with malachite green; remainder of the cells stained with Lugol iodine.

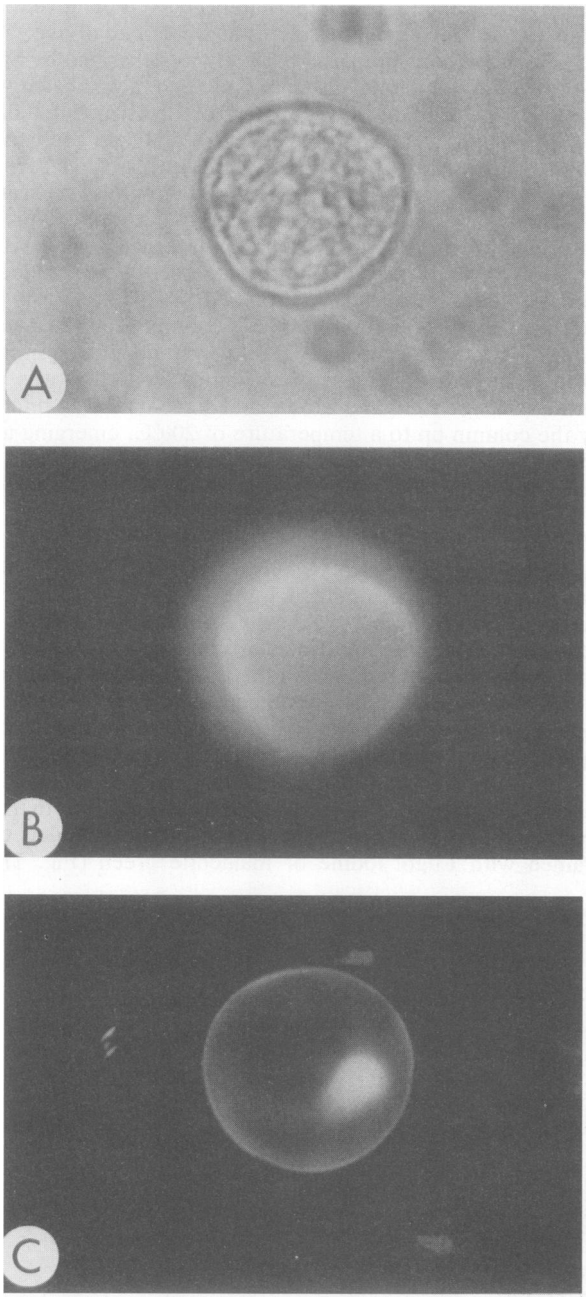

FIG. 2. Light and fluorescence microscopy of encysting cells of *E. histolytica*. (A) Cell stained with FITC-LPA in the light (×1,000) (B) Same cell under fluorescence. (C) Cell reacted with Calcofluor M2R under UV light (×1,000). Preincubation of the cysts and FITC-LPA lectin with sialic acid (0.2 M) inhibited the fluorescence. Trophozoites did not bind the LPA.

vealed multinuclear cells (approximately 20% of the population of cells), and many contained multiple chromatoid bodies. Trophozoites grown in Jones medium did not survive the ether treatment required for the malachite green staining, but when they were stained with the iodine stain, only a single nucleus could be seen clearly. A second nucleus was sometimes seen in some unusually large trophozoites which may have been in the process of division (<2%). The rounded cells from the magnesium sulfate medium became fluorescent when incubated with 0.1% Calcofluor M2R (Fig. 2). This stain has been used as a test for the presence of chitin and encystation in *E. invadens* (3). Cells that were treated with detergent (1% Vagisec) and were then returned to culture in Jones medium gave rise to viable cultures of trophozoites after 48 h in culture. On the basis of these criteria, the cells obtained from the magnesium sulfate medium were considered for the purpose of this study as encysting cells.

The encysting cells, but not trophozoites, bound FITC-LPA, a sialic acid-specific lectin (29), indicating a change in the sugars presented on the surface of the cells (Fig. 2). This binding was inhibited by preincubation of both the cells and the lectin with sialic acid (0.2 M). There was no binding to FITC-WGA by either population of cells, and no binding of the WGA lectin to nitrocellulose blots of the proteins was observed. Cysts obtained from a patient also displayed this pattern of lectin binding, i.e., they bound FITC-LPA, which could be inhibited by sialic acid, and they failed to bind FITC-WGA.

The profile of proteins stained by Coomassie brilliant blue on SDS-polyacrylamide gels of trophozoites and encysting cells differed quantitatively in a number of the protein bands (Fig. 3). In particular, a protein with a molecular size of about 150 kDa, which was also stained by Schiff reagent (Fig. 4), was present only in gels of preparations of encysting cells. Under the conditions used, no glycoproteins were stained by the Schiff reagent in preparations from trophozoites. No Schiff-reactive proteins in the 150-kDa region were seen in preparations of the bacteria which accompanied the trophozoites. Neither was there any Schiff-reactive protein in the 150-kDa range in the ox serum used for the encystation (Fig. 5). Preparations of the bacteria that were associated in the magnesium sulfate medium with the encysting cells displayed only a very faint trace of the 150-kDa protein which probably reflects disrupted amoeba (encysting) cell

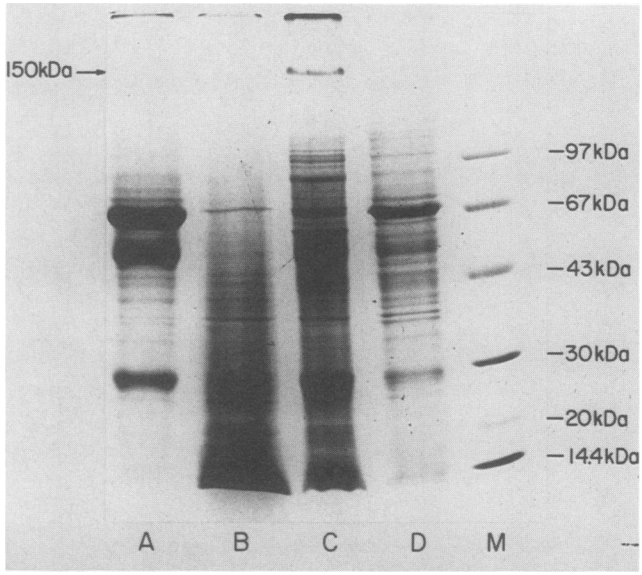

FIG. 3. SDS-PAGE on 7.5 to 15% acrylamide gradient gel of bacterial cells associated with the encysting cells (lane A), trophozoites (lane B), encysting cells (lane C), bacterial cells associated with the trophozoite cultures (lane D), and Pharmacia molecular size markers (lane M). The gel was stained with Coomassie brilliant blue, and 3×10^5 amoebal cells were loaded per lane. The amount of bacteria loaded (3×10^8 cells) was calculated to be equivalent to the total amount of bacteria in a culture containing this number of amoebae.

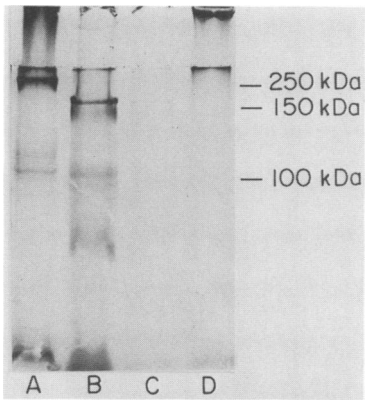

FIG. 4. SDS-PAGE on 7.5 to 15% acrylamide gradient gel of cysts of *E. invadens* (5) (lane A), encysting cells of *E. histolytica* (lane B), bacterial cells from cultures of encysting cells (lane C), and trophozoites of *E. histolytica* (lane D). Stained with Schiff reagent. The amounts loaded for each sample were as for Fig. 3.

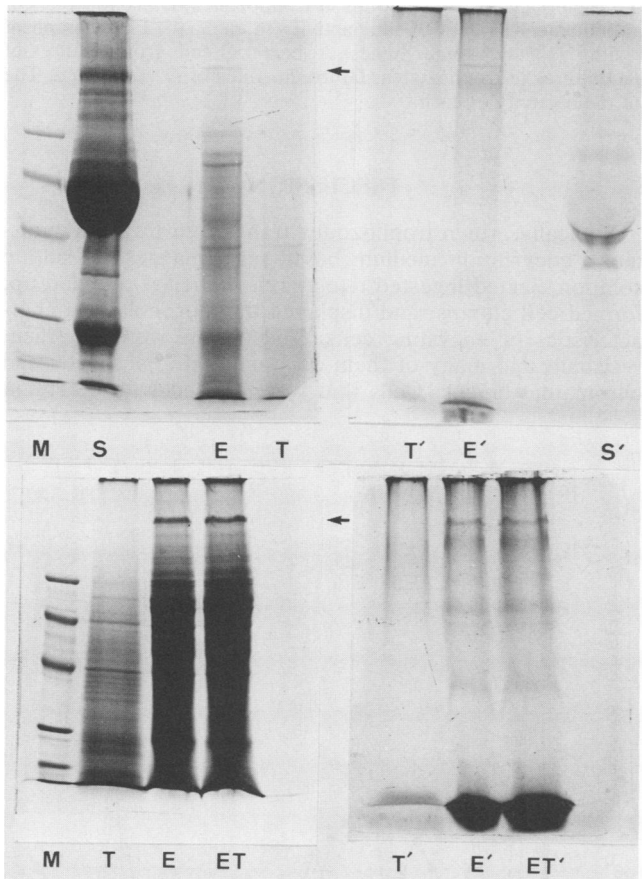

FIG. 5. SDS-PAGE of proteins from *E. histolytica* WI:0385:191 and from the ox serum used for the encystation. Upper panels: lane M, molecular weight markers; lane S, serum (6 μl); lane E, encysting cells (6×10^4); lane T, trophozoites (6×10^4); all lanes were stained with Coomassie brilliant blue. Lanes S′, E′, and T′ are the respective samples stained with the Schiff reagent. Lower panels: lane M, molecular weight markers; lane T, trophozoites; lane E, encysting cells; lane ET, encysting cells and trophozoites combined during the preparation of the gel samples; all lanes were stained with Coomassie brilliant blue. Lanes T′, E′, and ET′ are the respective samples stained with the Schiff reagent. Arrows show 150-kDa protein.

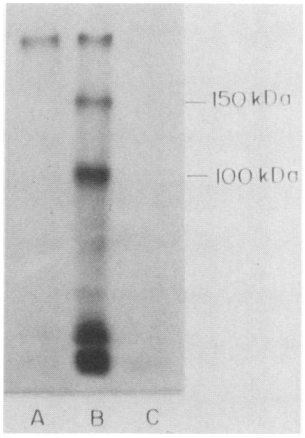

FIG. 6. Fluorography of SDS-PAGE on 7.5 to 15% acrylamide gradient gel of samples radiolabeled with $NaB[^3H]_4$ (see Materials and Methods). Lanes: A, trophozoites of *E. histolytica*; B, encysting cells of *E. histolytica*; C, control of encysting cells, no periodate. The amounts of samples loaded were as for Fig. 3.

components that cosedimented with the bacteria. In addition to the 150-kDa protein, a protein with molecular size of about 100 kDa was also faintly stained by the Schiff reagent, specifically in the encysting cells (Fig. 4). When trophozoites and encysting cells were mixed during the preparation and lysis for gel electrophoresis, no effect could be seen on the appearance of the 150-kDa protein band either by Coomassie brilliant blue or by Schiff staining (Fig. 5).

SDS-PAGE of trophozoites or encysting cells that were radiolabeled by reduction with tritiated borohydride after oxidation with periodate (1 mM) showed the same two proteins (150 and 100 kDa) as the only labeled proteins in preparations of the encysting cells (Fig. 6). Under the periodate oxidation conditions used, the sialic acids are preferentially labeled (17). No tritium-labeled proteins were detected on gels of trophozoite preparations. The radiolabeled cyst proteins were not significantly solubilized in a nonionic detergent (1% Nonidet P-40) nor as a result of treatment with high salt (0.5 M NaCl) or an ionic detergent (0.5% sodium deoxycholate). The only treatment that solubilized the proteins was boiling SDS (10%), indicating that they are tightly associated with the cells. Although some of the radiolabel was solubilized (Table 1) by the Nonidet P-40 and deoxycholate treatments, no protein bands were seen when this material was analyzed by SDS-PAGE.

Metabolic labeling of the cells with [^{35}S]methionine or [^{3}H]glucosamine showed that there was incorporation of the

TABLE 1. Solubilization of $NaB[^3H]_4$-labeled cyst material with salt and detergents[a]

Solvent	% of ^{3}H-labeled material solubilized
0.5 M NaCl	8[b]
0.5% Nonidet P-40	24[b]
1% Sodium deoxycholate	40[b]
10% SDS at 100°C	80

[a] Cells labeled with $NaB[^3H]_4$ were suspended in 10 mM Tris buffer (pH 8) and subjected to treatment with the salt or detergents for 15 min at 4°C and then centrifuged ($300 \times g$, 5 min). The pellet and supernatant were counted and also were prepared for and analyzed by SDS-PAGE.

[b] No protein bands were seen when this material was analyzed.

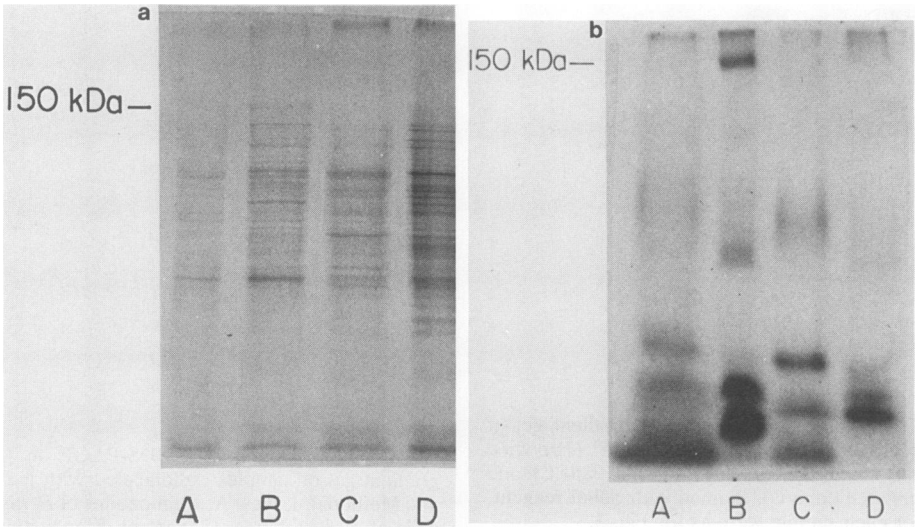

FIG. 7. (a) Fluorography of SDS-PAGE on 5 to 15% acrylamide gel of cells metabolically labeled with [^{35}S]methionine. Lanes: A, bacterial cells from cultures of encysting cells; B, encysting cells of *E. histolytica*; C, bacterial cells from cultures of trophozoites; D, trophozoites of *E. histolytica*. The amounts of samples loaded were adjusted to give an equivalent amount of radioactivity in each. (b) Fluorograph of SDS-PAGE on 7.5 to 15% acrylamide gel of cells metabolically labeled with [^{3}H]glucosamine. Lanes: A, bacterial cells from cultures of encysting cells; B, encysting cells of *E. histolytica*; C, bacterial cells from cultures of trophozoites; D, trophozoites of *E. histolytica*. The amounts of samples loaded were adjusted to give an equivalent amount of radioactivity in each.

label in both cases into the 150-kDa protein of the encysting cells. No label was incorporated into a protein of this size in the trophozoites or in the bacterial controls (Fig. 7a and b).

The cells labeled with [^{3}H]glucosamine contained radioactive, NaOH-insoluble material which was digestible (50%) by chitinase. Chromatograms of the chitinase digestion products (Fig. 8) revealed the presence of chitobiose (4, 26).

To investigate further the nature of the sugars labeled in the 150-kDa band, the glycoprotein was excised from nitrocellulose blots and the sugars were acid hydrolyzed and separated by TLC. Markers of sugars obtained under identical conditions were prepared from hydrolysates of fetuin and asialofetuin labeled with tritium and chromatographed as for those from the amoebal cells (Fig. 9). A comparison of the TLCs showed a peak of sialic acid that was released from the fetuin and that was missing in the asialofetuin. Hydrolysates of asialofetuin contained a second peak which, on the basis of the reported preference for the reaction (17), was probably galactose. Most of the labeled material hydrolyzed from the 150-kDa glycoprotein comigrated with the sialic acid peak hydrolyzed from fetuin (Fig. 9). Equivalent areas of gel excised and hydrolyzed from proteins of trophozoites did not give any peak of sialic acid.

Further proof that the sugars labeled with tritium in the 150-kDa protein include sialic acid was given by the observation that digestion with *V. cholera* sialidase released 75% of the radiolabel from the protein. This was compared with a control value of 8% released without enzyme. Fetuin under the same conditions gave values of 93 and 0.6% for the amounts of label released with and without sialidase present, respectively. When the material that was released by the sialidase was analyzed by TLC, it could be seen that the enzyme released material from the 150-kDa protein that comigrated with that released from radiolabeled fetuin (data not shown).

Electroeluted material that was analyzed by gas chromatography (Fig. 10) showed that the sugars included sialic acid with an estimated 15 residues per mol of 150-kDa protein.

DISCUSSION

Xenically grown trophozoites transferred to the hypoosmotic encystation medium based on a magnesium sulfate solution ejected ingested rice starch particles, rounded up, formed cell clumps, and displayed the morphological characteristics of encysting cells. These cells were detergent resistant, and many of them contained multiple nuclei and chromatoid bodies. Cells that were treated with detergent

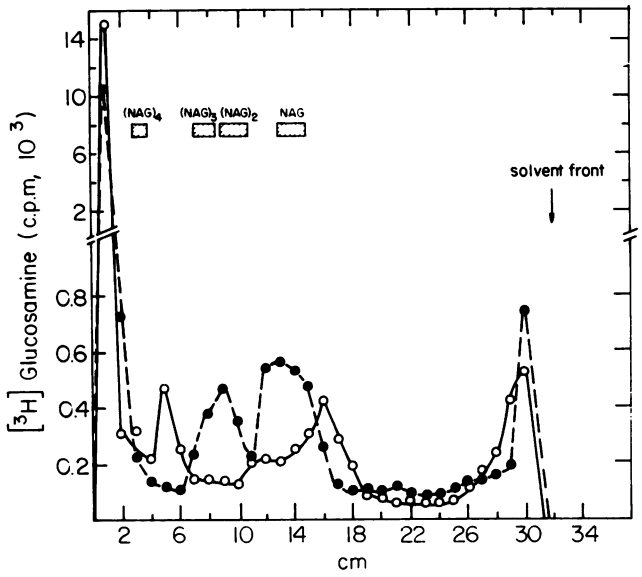

FIG. 8. Paper chromatograms of the [^{3}H]glucosamine-labeled cyst material remaining after treatment with NaOH (2 M, 1 h, 100°C) and digestion with chitinase (●). ○, Undigested material. The solvent used was *n*-butanol–acetic acid–water (4:1:5, vol/vol/vol, upper phase) (26). Markers used: NAG, *N*-acetylglucosamine; $(NAG)_2$, chitobiose.

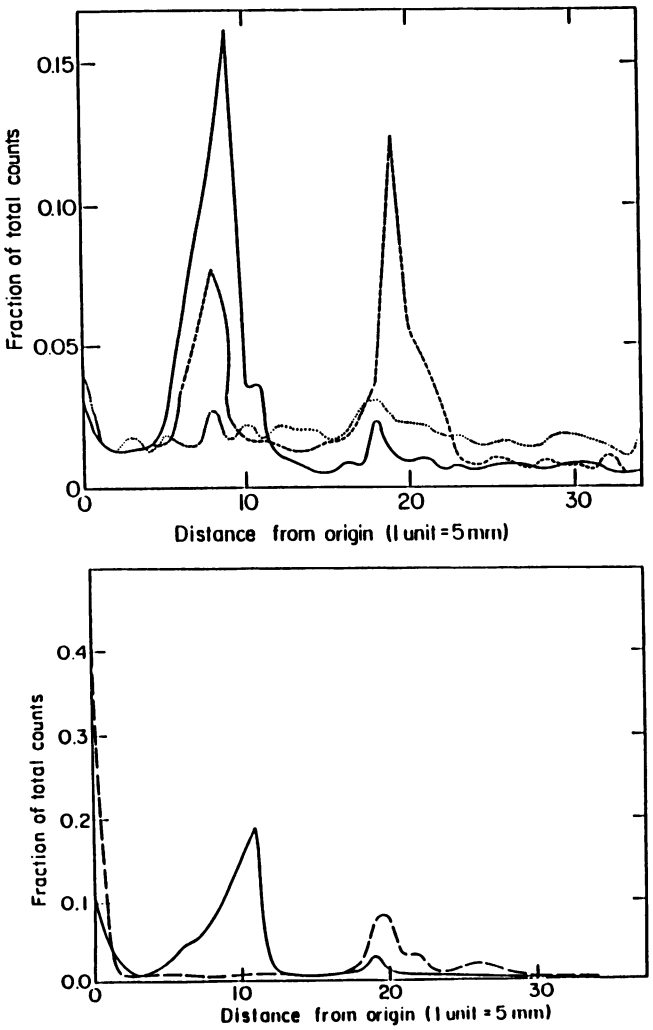

FIG. 9. Graphs of the results of TLCs of hydrolysates of proteins radiolabeled with $NaB[^3H]_4$ after periodate oxidation and excision from SDS-polyacrylamide gels. Upper panel: ——, Fetuin. ·····, 150-kDa area of SDS-PAGE of preparations of trophozoites of *E. histolytica*. ——, 150-kDa area of SDS-PAGE of preparations of encysting cells of *E. histolytica*. Lower panel: Comparison of hydrolysate products from ^3H-labeled fetuin (——) and asialofetuin (——).

and returned to growth medium gave rise to viable cultures of trophozoites. These cells may be more fairly compared to precysts of *E. invadens* than to mature cyst forms since most of them display neither typical characteristics of trophozoites nor the quadrinucleate cell morphology typical of mature cysts of *E. histolytica*.

The encysting cells of *E. histolytica* also differed from the trophozoites in the binding to the sialic acid-specific lectin LPA. This binding was inhibited by prior incubation with sialic acid. Cysts obtained from patients also bound FITC-LPA and resembled the cultured cysts too in that they failed to bind the lectin WGA. This is in contrast to the behavior of cysts of *E. invadens* (2, 3a), which bound WGA and not LPA. However, similar to what was observed with encysting cells of *E. invadens* (3a), in addition to the morphological changes, the cells transferred to the magnesium sulfate medium also displayed biochemical differences. Chitinase digestion of the cyst material labeled with [^3H]glucosamine which remained after NaOH treatment also showed that radiolabeled polymers of *N*-acetylglucosamine were obtained from the incorporated material.

Two new glycoproteins (150 and 100 kDa) that could be stained with Schiff reagent appeared in preparations of *E. histolytica* encysting cells. The 150-kDa protein in particular seems, from Coomassie blue staining, to be a major cell protein. The Schiff reagent used under these conditions did not stain any protein bands in preparations of trophozoites. This may reflect the availability of sugars to the reagent since trophozoites have been shown to contain a number of glycoproteins which can be detected with the lectin concanavalin A (1). The 150- and 100-kDa proteins were also the only ones to be labeled with tritiated sodium borohydride after oxidation with periodate. In this case, the labeling was under conditions that are known to oxidize and label sialic acid preferentially (17). That it was the sialic acids in these glycoproteins which became labeled was shown by the pattern of the hydrolysates run on TLC as well as by the observation that *V. cholera* sialidase digested a high percentage of the radiolabel. The sialidase-digested product comigrated with that digested from fetuin labeled under similar conditions. It is important to compare similarly treated material since the borohydride reduction chemically alters the structure of sialic acids on the glycoproteins.

Gas chromatography of the 150-kDa protein, on which we have focused attention, indicated that sialic acids were present with an estimated amount of 15 residues per mol of 150-kDa protein. This is comparable to the amount of sialic acid in fetuin.

The 150-kDa glycoprotein appears to be an integral component of the parasite cell membrane since it could not be solubilized by nonionic or ionic detergents or by high salt treatment. The metabolic labeling experiments indicate that it is synthesized during the incubation of cells in the hypoosmotic encystation medium. This makes it a differentiation antigen of the cells. Interestingly, it appears to be one of the main proteins that incorporate glucosamine in the amoebal cells (Fig. 7b). No Schiff-reactive protein in the region of 150 kDa was seen in the serum used in the encystation medium even when the full amount available to the same number of cells was compared. There is no other component in the encystation medium except the serum and $MgSO_4$. When trophozoites and encysting cells were mixed during the preparation and lysis for gel electrophoresis, the 150-kDa protein was not affected, which controls for the possibility that the more active proteases in the trophozoites degrade the protein so that it is only seen in the less active encysting cells. These findings show that the sialylated glycoproteins are not from the bacteria or the medium components.

We have previously reported (5, 12) the appearance of stage-specific sialylated glycoproteins in encysting cells of *E. invadens*, which are considered to be a model for *E. histolytica* cysts (3, 7, 11, 21, 22, 27). The glycoproteins we report here in *E. histolytica* encysting cells resemble somewhat those of *E. invadens* cysts in that they appear on the encysting cells, have a high- and lower-molecular-weight species, require the use of boiling ionic detergent to solubilize them, and contain sialic acid. The finding of sialic acid is in contrast to previous reports (16, 23) that *Entamoeba* species lack this sugar. However, those reports dealt with trophozoites and not with cysts, and our present results confirm the lack of sialic acid in proteins of trophozoites.

Despite the similarities, the sialylated glycoproteins from *E. invadens* and *E. histolytica* are biochemically and immunologically different. Cysts of *E. invadens* did not bind to FITC-LPA, although there was binding to WGA, which may

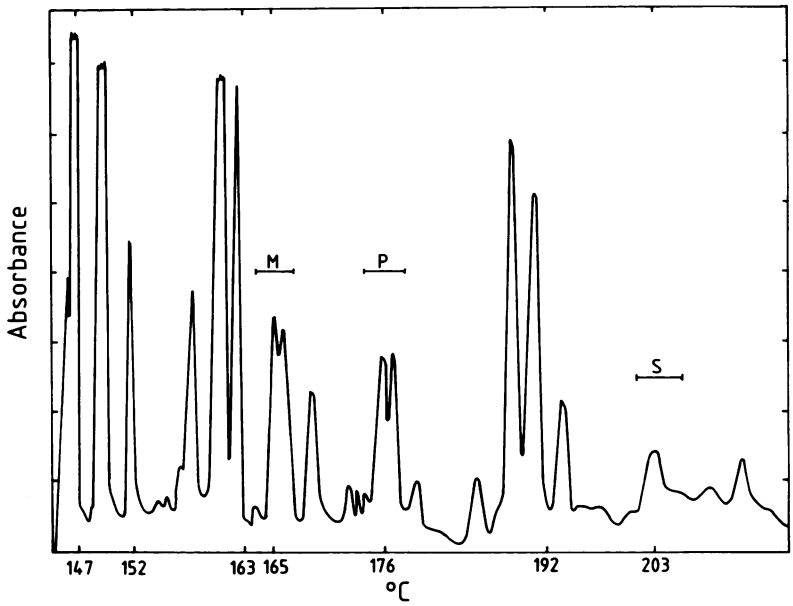

FIG. 10. Graph of gas chromatography of electroeluted 150-kDa protein. Chromatography was performed as described in the Materials and Methods. M, Mannitol; P, perseitol; S, sialic acid.

reflect differences in the mode of presentation of the sugars on the glycoproteins of the two species. Furthermore, none of five monoclonal antibodies that were prepared against the *E. invadens* sialoglycoprotein (3a) reacted with the encysting cells of *E. histolytica*. Neither did a polyclonal antiserum that was prepared against the *E. histolytica* 150-kDa protein cross-react with cysts or glycoproteins of *E. invadens* (data to be presented elsewhere). Monoclonal antibodies directed against the 150-kDa protein will be helpful in identifying any relation between the stage-specific sialylated glycoproteins in the two species of *Entamoeba* and may perhaps be useful for the diagnosis of cysts from human carriers.

The conditions of culture described do produce a good yield of cells that are biochemically and immunologically different from the trophozoites in a way that closely resembles the changes seen during the encystation of *E. invadens* (5, 12). Studies are under way to improve the yields of mature quadrinucleated cysts in these cultures and to verify the presence of immunologically related proteins present on cysts from patients harboring the parasite.

Possible reasons for the appearance of sialylated glycoproteins in encysting cells of *E. invadens* have been discussed elsewhere (3a). The present study suggests that this observation also holds for *E. histolytica*, which indicates again that the reptilian parasite is a good model for learning more about *E. histolytica* and also that sialylation may have a general function in the cyst stage of the *Entamoeba* parasite. In such a case, the culture system developed could be useful as a means to provide material for biochemical and immunological studies of at least the early stages in encystation of *E. histolytica*.

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