

Antigens in Electron-Dense Granules from *Entamoeba histolytica* as Possible Markers for Pathogenicity

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In vitro interaction of *Entamoeba histolytica* with collagen induces intracellular formation and release of electron-dense granules (EDG) and stimulation of collagenolytic activity. Purified EDG contain 1.66 U of collagenase per mg of protein. Thus, EDG may participate in tissue destruction during invasive amebiasis. Monoclonal antibodies (MAbs) L1.1 and L7.1 reacted specifically with EDG in enzyme-linked immunosorbent assay (ELISA) and immunofluorescence and immunoelectron microscopy. MAb L7.1 immunoprecipitated three polypeptides with molecular weights of 95,000, 68,000, and 28,000 from lysates of biosynthetically labeled *E. histolytica*. Both MAbs recognized the pathogenic *E. histolytica* axenic strains HM1:IMSS, HM38:IMSS, and HK-9 but failed to react in ELISA with *Entamoeba moshkovskii*, *Entamoeba invadens*, and *E. histolytica*-like Laredo. In addition, MAb L7.1 reacted with one *E. histolytica* isolate from a symptomatic patient but did not react with four of five isolates from asymptomatic patients. EDG antigens were detected by a MAb L7.1-based ELISA in *E. histolytica*-containing fecal samples from symptomatic, but not asymptomatic, individuals. These results suggest that the EDG antigen detected with MAb L7.1 may be differentially expressed in pathogenic and nonpathogenic *E. histolytica*.

Invasive amebiasis, a disease caused by the enteric protozoan *Entamoeba histolytica*, is a major public health problem in developing countries (33). Recently, progress has been made in understanding the molecular basis of the pathogenesis of this disease. Thus, it has been shown that attachment of *E. histolytica* trophozoites to mammalian cells, which is mediated by a surface lectin, is required for direct-contact amebic cytolytic activity (20-22). An amebic protein which forms ion channels in the membranes of target cells and probably participates in cytotoxicity has also been identified (15, 34). In addition, proteolytic enzymes present on the parasite surface have been implicated in the disruption of the intestinal extracellular matrix (8, 14, 18, 19, 24).

Since collagen is a major component of the extracellular matrix, in previous studies we have focused our attention on the *E. histolytica* collagenase (16, 18, 19, 24). The levels of this enzyme correlate with the virulence of different *E. histolytica* strains (8, 19). The collagenase is specific for type I collagen and is localized in the plasma membrane of the trophozoite. In addition, the collagenolytic activity in trophozoites increases when they are cultured in the presence of collagen (16). In recent *in vitro* studies, we have shown that the increase in collagenolytic activity is accompanied by the intracellular formation of electron-dense granules (EDG). These granules accumulate in the parasite plasma membrane and are subsequently released into the extracellular milieu (16). The significance of these observations is, however, obscure.

To gain insight into the relationship of EDG formation and release with collagenolytic activity and pathogenicity, we produced monoclonal antibodies (MAbs) that specifically

recognize EDG components. These MAbs were used to analyze the cellular distribution of EDG antigens and investigate their presence in pathogenic and nonpathogenic strains of *E. histolytica*. In addition, a MAb-based enzyme-linked immunosorbent assay (ELISA) for fecal samples was developed to search for EDG antigens in patients with symptomatic and asymptomatic amebiasis.

MATERIALS AND METHODS

Parasites. *E. histolytica* HM1:IMSS, HM38:IMSS, and HK-9 were cultured axenically in TYI-S-33 medium (7) at 37°C. *Entamoeba moshkovskii* FIC, *E. moshkovskii* CST, *Entamoeba invadens*, and *E. histolytica*-like Laredo were cultivated in the same medium at 25°C. *E. histolytica* HK-9, *E. moshkovskii* CST, and the Laredo isolate were kindly provided by L. S. Diamond (National Institutes of Health, Bethesda, Md.). Axenic *E. histolytica* HM1:IMSS, HM38:IMSS, and HK-9, having pathogenic-type zymodemes, were originally isolated from patients with active amebic disease. *E. invadens* is a species which infects reptiles, whereas *E. moshkovskii* CST and FIC, originally isolated from sewage water, have no known host. *E. histolytica*-like Laredo is generally considered a species different from *E. histolytica* and taxonomically closer to *E. moshkovskii* (1).

Five *E. histolytica* isolates (HM42:IMSS, HM43:IMSS, HM44:IMSS, HM46:IMSS, and HM47:IMSS) from stools of asymptomatic carriers (17) and one isolate (HM48:IMSS) from a patient with amebic dysentery were all grown in xenic cultures in modified (5) Boch and Drbohlav medium. Isolates HM42:IMSS to HM47:IMSS, having nonpathogenic zymodemes (17), were obtained from asymptomatic donors showing no clinical, serological, or endoscopic signs of amebic intestinal invasion (17).

Collagenase assay. Human collagen type I was extracted

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from placentas as described previously (18). For the collagenase assay, samples of type I collagen (20 μ l; 3 mg/ml) in 0.05 M Tris hydrochloride buffer (pH 7.2) were added to each well of tissue culture multiwell plates (model 76-003-05; Linbro, Chemical Co., Hamden, Conn.). Plates were incubated at 37°C for 1 h, and then after sterilization with UV light, the collagen films were incubated for 3 h at 37°C with EDG (10, 100, 200, and 400 μ g of protein) suspended in Ringer solution containing 5.5 μ g of Carbecin (a gift of Laboratorios Sanfer, México, D.F., Mexico) per ml. After incubation, the films were fixed in 2.5% (wt/vol) glutaraldehyde, washed, and stained with picosirius solution (0.1% sirius red in saturated aqueous picric acid) for 1 h (10). Excess dye was washed out three times with 0.1 N HCl, and the dye bound to collagen was eluted with 200 μ l of 0.01 N NaOH in absolute methanol (1:1). Then, samples diluted 1:10 were read in a spectrophotometer at 540 nm. A unit of collagenase was defined as the enzymatic activity required to digest 1 μ g of collagen per min at 37°C (18).

Induction of EDG release by collagen. Trophozoites of *E. histolytica* HM1:IMSS (10^6 /ml) were suspended in TYI-S-33M medium (18) containing collagen type I (0.3 mg/ml) and Carbecin (5.5 μ g/ml). After incubation for 16 h at 37°C, cells were chilled (4°C) and collected by centrifugation ($230 \times g$ for 5 min). Supernatant containing released EDG was centrifuged for 10 min at $650 \times g$ to remove debris, and then EDG were pelleted at $13,000 \times g$ for 15 min and suspended in Ringer solution. Protein was determined by the Lowry assay (13), using bovine serum albumin as a standard.

Monoclonal and polyclonal antibodies. The EDG-rich pellet was used as the antigen to produce MAbs in mice. BALB/c mice were subcutaneously injected with 150 μ g of EDG protein in 300 μ l of phosphate-buffered saline (PBS) emulsified with 300 μ l of complete Freund adjuvant. Eighteen days later, the mice were intraperitoneally inoculated with 150 μ g of antigen in PBS, and three days after their spleens were removed for fusion. Cell fusion was carried out with polyethylene glycol by standard procedures (11) using cell line SP2/0-Ag14 (25). Initial screening of culture supernatants was performed by ELISA (32), using *E. histolytica* HM1:IMSS total antigen. Purified EDG was used as the antigen for the secondary screening of supernatants. Cells were subcloned by limiting dilution and two hybridoma lines (L1.1 and L7.1) were obtained. Both MAbs were of the immunoglobulin M (IgM) class. They were used either as culture supernatants or in ascites form with identical results. Because hybridoma L7.1 is a better antibody secretor, it was chosen for most experiments.

Anti-EDG polyclonal antibodies were prepared from the serum of a goat hyperimmunized with EDG. BALB/c mouse antiserum to strain HM1:IMSS was produced by two intraperitoneal immunizations with 2×10^6 live trophozoites each.

Immunoprecipitation. Five million *E. histolytica* trophozoites from logarithmic-phase cultures were incubated for 6 h in 3 ml of serum-free TYI-S-33 medium containing 600 μ Ci of [35 S]methionine (1209 Ci/mmol; New England Nuclear, Boston, Mass.). Trophozoites were harvested, washed twice with cold PBS containing 0.27 M NaCl, and solubilized by vortexing in 40 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM *p*-hydroximercuribenzoate (PHMB). Samples were stored at -70°C until used. Insoluble material was removed from the lysate before immunoprecipitation by centrifugation at $16,000 \times g$ for 10 min at 4°C. For immunoprecipitation, the IgM MAb L7.1

was bound to affinity-purified rabbit anti-mouse IgM previously linked to protein A-Sepharose beads (Pharmacia Fine Chemicals, Piscataway, N.J.) as described by Yuan et al. (35). Fifty microliters of rabbit anti-mouse IgM-protein A-Sepharose was incubated for 4 h at 4°C with continuous rotation, with 100 μ l of a 1-mg/ml solution of MAb L7.1. Thereafter, the Sepharose beads were washed twice with PBS containing 0.5% Triton X-100, 2 mM PMSF, and 1 mM PHMB and incubated for 3 h at 4°C with 100 μ l of lysate containing 5×10^5 labeled amebas. After incubation, beads were washed twice with PBS containing 0.5% Triton X-100, and after an additional wash with PBS, the beads were boiled in 100 μ l of 4 \times sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and centrifuged, and the supernatant was loaded on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis Laemmli gels (12). After electrophoresis, the gels were fixed and processed for fluorography (2).

Immunofluorescence. Trophozoites were fixed for 15 min with 1.85% formaldehyde and 0.125% glutaraldehyde at 37°C, washed with PBS, and incubated with 1 M glycine for 15 min at 37°C. The cells were then permeabilized with 0.3% (wt/vol) Triton X-100 in 15 mM phosphate buffer (pH 7.2) containing 180 mM NaCl and washed three times with the same solution. They were then incubated with 0.0025% Evans blue for 20 min at 37°C and finally treated with MAb L1.1 or L7.1 (ascites fluid diluted 1:50) overnight at 4°C. After being washed with PBS, cells were incubated with fluorescein-conjugated goat anti-mouse IgM (Hyclone Laboratories, Inc., Utah). Excess second antibody was removed by washing three times with PBS. Trophozoites were then mounted in 50% glycerol and observed by fluorescence microscopy.

Immunoelectron microscopy. Trophozoites, incubated for 6 h with collagen (0.3 mg/ml), were fixed in a mixture of 0.125% glutaraldehyde and 1.85% formaldehyde in PBS for 15 min at 37°C. After permeabilization with 0.3% Triton X-100, the cells were washed and then incubated with 20 μ l of ascites fluid containing MAb. Cells were washed three times in PBS and incubated for 30 min at room temperature with 50 μ l of rabbit anti-mouse IgM diluted 1:200. After three PBS washings, cells were incubated with 10 μ l of protein A-colloidal gold diluted 1:2. Cells were finally fixed with 2.5% glutaraldehyde and processed for electron microscopy (31). Negative controls included normal ascites fluid or omission of MAb.

Stool samples. Stool specimens from children known to be positive for *E. histolytica* by microscopic stool examination were collected at the Hospital Infantil in Mexico City. Specimens negative for *E. histolytica* by more than three microscopic examinations were used as controls. Before being assayed, a sample of stool specimens (approximately 100 μ l) was diluted in 1 ml of PBS containing 0.05% Tween-20 (PBS-T).

ELISA for fecal samples. The method used was a modification of the double-antibody ELISA described by Ungar et al. (29, 30) and Del Muro et al. (6). All washing procedures were performed three times with PBS-T. Wells of polystyrene ELISA plates (Immulon I; Dynatech Laboratories, Alexandria, Va.) were coated with goat anti-EDG antibodies (100 μ g/0.1 ml) in 0.1 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. Remaining uncoated sites were blocked with 1% bovine serum albumin in PBS-T, and the wells were washed as described above. Fecal samples in PBS-T (50 μ l) were added and incubated for 2 h at 37°C and washed as described above. To detect EDG antigen bound to

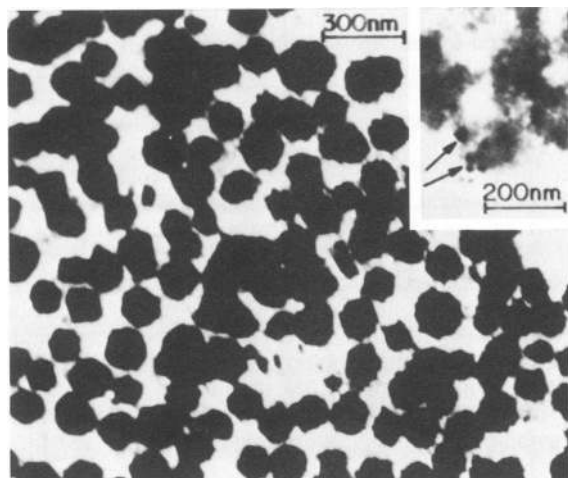


FIG. 1. Transmission electron microscopy of an EDG preparation obtained from *E. histolytica* HM1:IMSS. The inset shows an immunoelectron microscopic analysis of the same EDG preparation stained with MAb L7.1, followed by rabbit anti-mouse IgM and gold-labeled protein A. The arrows point to gold particles.

the polyclonal antibodies on the wells, these were incubated for 1 h at 37°C with MAb L7.1 diluted 1:1,000 in PBS-T with 1% bovine serum albumin. Plates were washed and 50 μ l of a 1:1,000 dilution of peroxidase-conjugated goat anti-mouse immunoglobulin (Zymed Laboratory, South San Francisco, Calif.) was added. After incubation for 1 h at 37°C, plates were washed again and 100 μ l of orthophenylenediamine in H₂O₂ was added and allowed to react in the dark for 30 min at room temperature.

A stool sample was considered positive if the optical density reading was higher than the arithmetic mean plus 2 standard deviations of the optical density of 25 *E. histolytica*-negative control samples.

RESULTS AND DISCUSSION

EDG are enriched in collagenase. We have previously shown that incubation of trophozoites with collagen induces the formation of EDG which accumulate in the parasite plasma membrane and are subsequently released into the extracellular medium (16). Concomitantly, there was a large increase in plasma membrane-bound collagenolytic activity associated with degradation of collagen. Therefore, we hypothesized that EDG are related to the lytic properties of the trophozoites and that they might contain collagenase (16, 24). To test this possibility, a preparation highly enriched in EDG was obtained from supernatants of trophozoites cultured in the presence of collagen. By transmission electron microscopy, no structures other than EDG were observed in such preparation (Fig. 1). When the collagenolytic activity of the purified EDG was measured by using collagen type I as a substrate, 1.66 U of collagenase were detected per mg of EDG protein. This specific activity is approximately 10- and 24-fold higher than that found in whole trophozoites incubated with and without collagen, respectively. These results suggest that EDG participate in the packing and secretion of collagenase and therefore may play a role in tissue destruction during invasive amebiasis.

MAbs against EDG. To further characterize EDG, we prepared MAbs against them. Of the 18 clones that secreted antibodies reacting with total *E. histolytica* antigen, two IgM

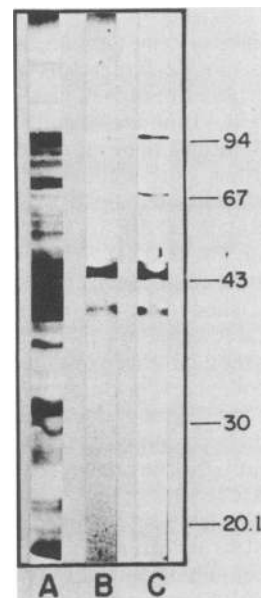


FIG. 2. Immunoprecipitation of *E. histolytica* HM1:IMSS antigens biosynthetically labeled with [³⁵S]methionine. Lane A, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of whole trophozoites labeled with [³⁵S]methionine; lane B, polypeptides precipitated with rabbit anti-mouse IgM bound to protein A-Sepharose; lane C, polypeptides precipitated with MAb L7.1 attached to rabbit anti-mouse IgM-protein A-Sepharose (a specifically precipitated 28,000-Da polypeptide present in the original fluorograph was very faint and cannot be seen in this photograph).

(MAbs L1.1 and L7.1) that also bound to EDG-coated ELISA plates were selected. Their reaction with purified EDG was also demonstrated by immunoelectron microscopy (Fig. 1, inset). MAb L7.1 was used for most experiments, because it could be obtained in larger quantities than L1.1.

Our initial attempts to determine the nature of the antigen recognized by the MAb using the immunoblotting technique (28) were unsuccessful. However, by immunoprecipitating *E. histolytica* HM1:IMSS antigens (that had been metabolically labeled with [³⁵S]methionine) with MAb L7.1, we could detect the specific precipitation of three polypeptides having molecular weights of 95,000, 68,000, and 28,000 (Fig. 2). The 95,000- and 68,000-Da polypeptides were the most prominent in the fluorograph, whereas the 28,000-Da band was very faint. Two additional polypeptides of 45,000 and 40,000 Da were nonspecifically precipitated and also seen in control immunoprecipitates without MAbs. Because of the limitations of the immunoprecipitation technique, we do not know if all three specifically precipitated polypeptides bear the epitope recognized by MAb L7.1. Presumably, this MAb is not directed to collagenase because the molecular weights of the immunoprecipitated polypeptides do not agree with that we have recently found for collagenase (unpublished observations). In addition, MAb L7.1 failed to inhibit the collagenolytic activity of trophozoites (data not shown).

To further understand the events leading to the assembly of EDG, MAbs L1.1 and L7.1 were used to examine the cellular distribution of the EDG antigens in trophozoites incubated with and without collagen. Both MAbs yielded identical staining patterns by indirect immunofluorescence. Fluorescence was localized mostly in the membrane of trophozoites incubated without collagen, and more than 90%

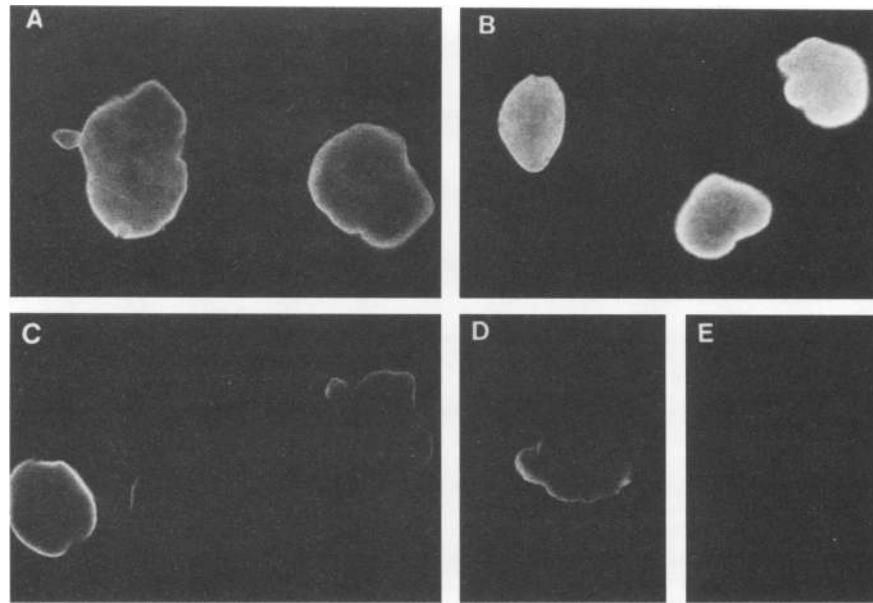


FIG. 3. Immunofluorescence microscopy of *E. histolytica* HM1:IMSS trophozoites stained with anti-EDG MAb L7.1. (A) Trophozoites cultured without collagen and stained with MAb L7.1; (B to D) trophozoites incubated with collagen for 3 (B), 6 (C), or 16 (D) h; (E) trophozoites incubated with collagen (16 h) and stained with nonspecific mouse immunoglobulin.

of these were labeled (Fig. 3A). After 3 h of incubation in liquid medium containing collagen, a similar staining pattern was observed and fluorescence was increased (Fig. 3B). After 6 h of incubation, the number of labeled trophozoites decreased to approximately 70% and fluorescence was localized in the peripheral cytoplasm and plasma membrane (Fig. 3C). After 16 h of incubation with collagen, trophozoites showed significantly diminished membranous and cytoplasmic fluorescence and only an estimated 10% of the cells were

labeled (Fig. 3D). Trophozoites treated with nonspecific mouse immunoglobulin or with fluorescein-labeled second antibody alone showed no fluorescence (Fig. 3E). Colloidal gold immunoelectron microscopy studies using MAb L7.1 confirmed and extended the immunofluorescence findings. Gold particles were found only in the plasma membrane of trophozoites not incubated with collagen (Figure 4A). EDG were not observed. In contrast, labeled EDG were found extracellularly, in the cytoplasm, and beneath the plasma

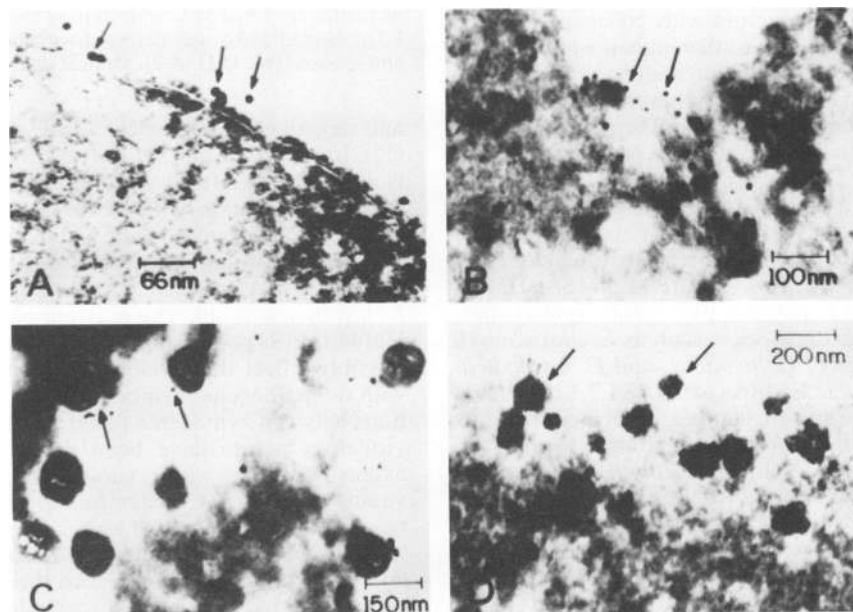


FIG. 4. Colloidal gold immunoelectron microscopy of *E. histolytica* HM1:IMSS trophozoites. (A) Trophozoites cultured without collagen; (B to D) trophozoites incubated with collagen for 6 h. The cells were fixed and treated with MAb L7.1, followed by rabbit anti-mouse IgM and gold-labeled protein A. Note the gold particles (arrows) in electron-dense areas close to microtubule-like structures (B) and in electron-dense granules (C and D).

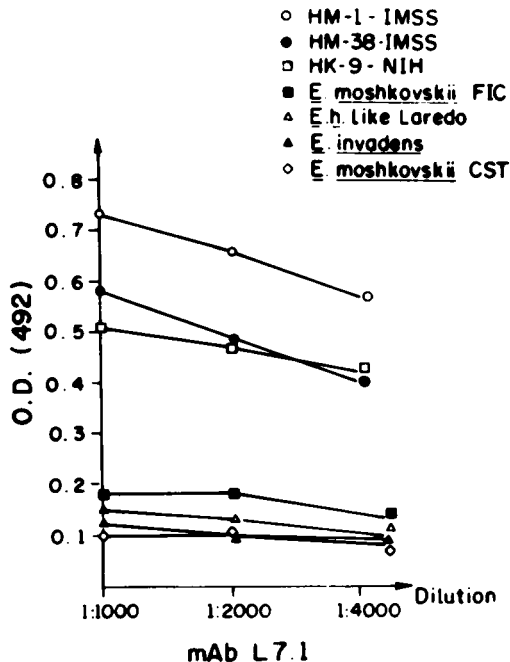


FIG. 5. Binding of MAb L7.1 to antigens from different strains of *E. histolytica* and other *Entamoeba* species. ELISA wells coated with 10 μ g of *Entamoeba* antigen were incubated for 1 h at 37°C with 50 μ l of the indicated dilution of MAb. Bound MAb was detected with peroxidase-conjugated goat anti-mouse IgM. In this assay, mouse polyclonal antibodies against *E. histolytica* HM1:IMSS strain cross-reacted with all *Entamoeba* species tested.

membranes of trophozoites incubated for 6 h with collagen (Fig. 4C and D). Dispersed labeling of plasma membrane was also observed. Gold particles in these trophozoites were also found in electron-dense zones close to microtubulelike structures (Fig. 4B). In summary, these studies revealed that the EDG antigen detected by the MAb is redistributed in the trophozoites after their interaction with collagen. Furthermore, after prolonged incubation, the antigen was lost from trophozoites, as evidenced by lack of staining in immunofluorescence. This antigen loss is due presumably to the release of EDG into the extracellular medium. These findings indicate that molecules initially present in the plasma membrane are incorporated into EDG. However, the data does not allow the site of EDG assembly to be precisely located.

Strain specificity of MAbs. Reactivity of MAbs L1.1 and L7.1 was tested against antigens from trophozoites of three axenic strains of *E. histolytica* (HM1:IMSS, HM38:IMSS, and HK-9) which possess pathogenic zymodemes, and from other *Entamoeba* species, such as *E. moshkovskii* FIC, *E. moshkovskii* CST, *E. invadens*, and *E. histolytica*-like Laredo. Results of the ELISAs for MAb L7.1 are shown in Fig. 5. MAb L7.1 showed identical specificity. MAbs recognized only the three *E. histolytica* strains. In contrast, mouse polyclonal antibodies to *E. histolytica* HM1:IMSS cross-reacted with all *Entamoeba* species (data not shown). MAb L7.1 was also tested against *E. histolytica* clinical isolates maintained in xenic cultures (Fig. 6). One isolate (HM48:IMSS) from a patient with clinical symptoms of amebiasis gave a strong positive reaction in the ELISA. In contrast, of five additional isolates obtained from asymptomatic carriers, only one (HM44:IMSS) was positive. These five cultures have nonpathogenic zymodemes and were isolated from individuals free of gastrointestinal symptoms

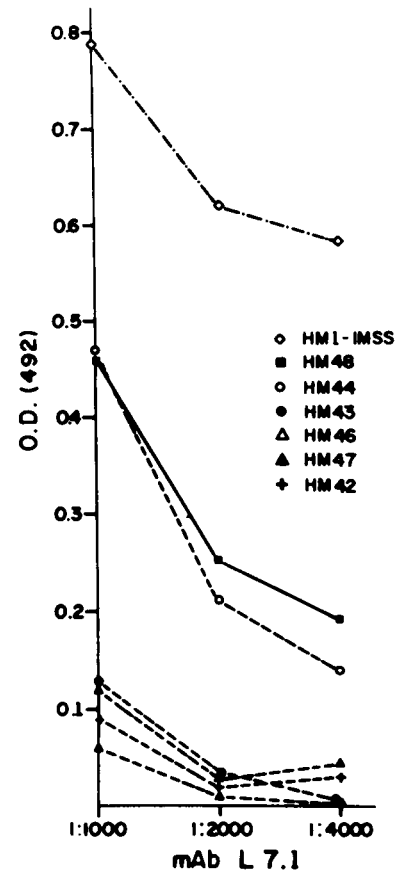


FIG. 6. Binding of MAb L7.1 to *E. histolytica* clinical isolates. Axenic strain HM1:IMSS, isolate HM48:IMSS from a patient with symptoms of amebiasis, and isolates (HM42:IMSS, HM43:IMSS, HM44:IMSS, HM46:IMSS, and HM47:IMSS) with nonpathogenic zymodemes from asymptomatic individuals. For the assay, ELISA wells coated with 10 μ g of antigen from the different isolates were incubated for 1 h at 37°C with 50 μ l of the indicated dilution of MAb L7.1. Bound MAb was detected with peroxidase-conjugated goat anti-mouse IgM. O.D.(492), Optical density at 492 nm.

and negative in serological assay and rectosigmoidoscopy (17). In summary, all *E. histolytica* axenic strains and clinical isolates obtained from invasive cases of amebiasis exhibited a positive reaction with MAb L7.1, whereas most of the isolates from asymptomatic patients were negative. These results suggest that expression of the epitope recognized by MAb L7.1 may correlate with the pathogenic potential of the *E. histolytica* strain. The reaction of MAb L7.1 with isolate HM44:IMSS possessing nonpathogenic zymodemes may possibly reflect the variability in virulence levels of isolates with nonpathogenic zymodemes (4). Exceptions in correlations between zymodeme pattern and expression of putative virulence factors have been noted by others (23). More extensive studies with a variety of additional *E. histolytica* strains are planned to determine the relationship between reactivity with MAb L7.1 and pathogenicity.

Detection of EDG components in stool samples. The above findings prompted us to explore the feasibility of using the anti-EDG MAb to detect *E. histolytica* in stool samples. MAb L7.1 was used in combination with goat polyclonal anti-EDG antibodies to develop a double-antibody ELISA. Twenty-one stool specimens in which *E. histolytica* cysts were demonstrated by microscopic examination were ana-

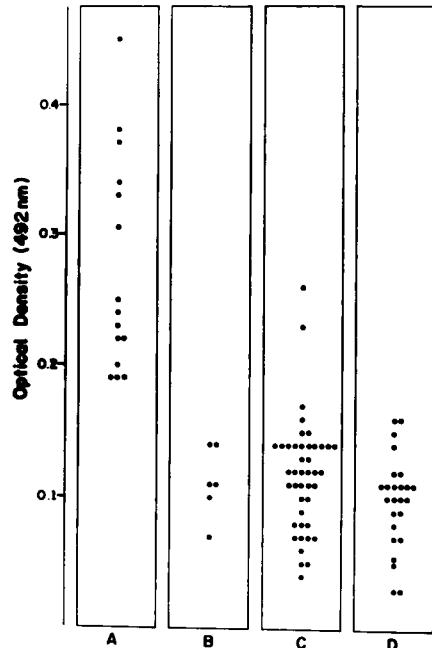


FIG. 7. Results of double-antibody ELISA for detection of *E. histolytica* in fecal specimens. (A) Fifteen *E. histolytica*-positive samples from symptomatic individuals; (B) six *E. histolytica*-positive samples from asymptomatic individuals; (C) forty-three samples containing other parasites; and (D) twenty-five samples without parasites. This assay detects as little as 50 ng of *E. histolytica* HM1:IMSS antigen. The anti-EDG polyclonal antibodies used to coat the ELISA wells are specific for *E. histolytica* species.

lyzed by this ELISA (Fig. 7). Of the 21 specimens, 15 were positive. These 15 positive samples were obtained from patients with classical amebic dysentery symptoms. In contrast, the six ELISA-negative stool samples came from asymptomatic individuals in whom cysts were incidentally found during routine microscopic stool examinations. These negative results cannot be explained by the paucity of trophozoites in stools of asymptomatic carriers because, as demonstrated by indirect immunofluorescence tests, the antigen detected by MAb L7.1 is present in cysts from symptomatic, but not in those from asymptomatic, individuals (data not shown). Sixty-eight fecal samples without demonstrable *E. histolytica* were examined as specificity controls for the ELISA. Forty-three of these samples contained other parasites such as *Entamoeba coli*, *Endolimax nana*, *Ascaris lumbricoides*, *Trichuris trichiura*, *Hymenolepis nana*, and *Giardia lamblia*. All 25 parasite-free samples, as determined by microscopy, yielded negative results for *E. histolytica* in the ELISA (Fig. 7). Of the 43 samples containing parasites other than *E. histolytica*, 41 also yielded negative results in the ELISA. The finding of positive ELISA readings in two samples apparently devoid of *E. histolytica* may reflect a higher sensitivity of ELISA compared with microscopy for the detection of intestinal amebiasis (6, 29, 30).

These results further suggest that MAb L7.1 may identify invasive strains of *E. histolytica*. Differences between pathogenic and nonpathogenic *E. histolytica* isolates have been observed recently using MAbs (26) and DNA probes (3, 9, 27). Our MAbs against EDG may be an additional tool to investigate the basis for *E. histolytica* pathogenicity.

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