Protection against Invasive Amebiasis by a Single Monoclonal Antibody Directed against a Lipophosphoglycan Antigen Localized on the Surface of Entamoeba histolytica

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Summary

A panel of monoclonal antibodies was raised from mice immunized with a membrane preparation from Entamoeba histolytica, the pathogenic species causing invasive amebiasis in humans. Antibody EH5 gave a polydisperse band in immunoblots from membrane preparations from different E. histolytica strains, and a much weaker signal from two strains of the nonpathogenic species Entamoeba dispar. Although the exact chemical structure of the EH5 antigen is not yet known, the ability of the antigen to be metabolically radiolabeled with [32P]phosphate or [3H]glucose, its sensitivity to digestion by mild acid and phosphatidylinositol-specific phospholipase C, and its specific extraction from E. histolytica trophozoites by a method used to prepare lipophosphoglycans from Leishmania showed that it could be classified as an amebal lipophosphoglycan. Confocal immunofluorescence and immunogold labeling of trophozoites localized the antigen on the outer face of the plasma membrane and on the inner face of internal vesicle membranes. Antibody EH5 strongly agglutinated amebas in a similar way to concanavalin A (Con A), and Con A bound to immunoaffinity-purified EH5 antigen. Therefore, surface lipophosphoglycans may play an important role in the preferential agglutination of pathogenic amebas by Con A. The protective ability of antibody EH5 was tested in a passive immunization experiment in a severe combined immunodeficient (SCID) mouse model. Intrahepatic challenge of animals after administration of an isotype-matched control antibody or without treatment led to the development of a liver abscess in all cases, whereas 11 out of 12 animals immunized with the EH5 antibody developed no liver abscess. Our results demonstrate the importance and, for the first time, the protective capacity of glycan antigens on the surface of the amebas.

The intestinal parasite Entamoeba histolytica is a major cause of human morbidity and mortality, claiming up to 100,000 victims every year (1). Entamoeba dispar is its nonpathogenic counterpart, morphologically very similar, but defined as a separate species (2). The molecules on the surface of the amebas have been studied extensively because they interact with the human host and also represent possible vaccine candidates. In 1991, Espinosa-Cantallano and Martinez-Palomo reviewed the characterization of a number of surface proteins of E. histolytica (3), the most important being the galactose- and N-acetylgalactosamine-inhibitable lectin (4-6), which the amebas use to adhere to host cells and other target structures. Since then more data about the structure (for example see reference 7) and functional properties (reference 8) of known surface proteins have been obtained, and more surface proteins have been described (9, 10). The galactose- and N-acetylgalactosamine-inhibitable lectin (11-13), as well as the serine-rich surface protein (14) and the 29-kD putative surface antigen (15), were suggested as vaccine candidates and were tested in rodent models.

Lipophosphoglycan antigens from E. histolytica were described for the first time by Isibasi et al. (16), and later monoclonal antibodies (17-20) have been used to study
their expression under different culture conditions (21) and in different E. histolytica strains (22). One antibody against lipophosphoglycans was able to inhibit adhesion of amebas to target cells and cytotoxicity (20). Recently, the expression of lipophosphoglycans was correlated to amebic virulence (23).

In this report we describe a new antibody, EH5, that preferentially bound to E. histolytica strains and much less to E. dispar. We demonstrated that the EH5 antigen was a lipophosphoglycan and for the first time localized the antigen on the outer face of the plasma membrane and the inner face of internal vesicles by confocal immunofluorescence and immunoelectron microscopy. The immunofinity-purified antigen bound to Con A and may be important for the effect of agglutination of E. histolytica by Con A. The EH5 antibody significantly protected SCID mice against intrahepatic challenge with E. histolytica. Taking all this together, we show that lipophosphoglycans are major protective antigens on the surface of pathogenic amebas.

Materials and Methods

Strains and Growth Conditions. Trophozoites of the pathogenic E. histolytica strains SFL-3, HM-1:IMSS, 200:NIH, and HK-9 were cultured axenically at 37°C in TYI-S-33 medium (24). E. dispar strain SAW760 (25) was maintained monoxenically in TYI-5-33 medium (24). E. histolytica strain SAW142 was cultured axenically in TYSGM-9 medium (26). E. histolytica strain SFL-3 was also cultured axenically in N robinson medium (27). Trichomonas vaginalis strain 30001 (American Type Culture Collection, Rockville, MD) was grown in TYM medium (28) supplemented with 5% (vol/vol) heat-inactivated horse serum and 0.05% (wt/vol) agar.

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Membrane Antigen Preparation. Membrane antigens were prepared as described by Ramanwani and Mishra (29). In brief, trophozoites were harvested by centrifugation, washed three times with 150 mM NaCl, and finally resuspended in 100 mM sodium phosphate, 1 mM EDTA, and 5 mM iodoacetamide, pH 7.2. The trophozoites were homogenized by 30 strokes in a Dounce homogenizer, and debris was precipitated by ultracentrifugation at 100,000 g for 1 h and 4°C. Membrane antigens were precipitated by ultracentrifugation at 100,000 g at 4°C for 1 h and resuspended in distilled water or the desired buffer.

Immunization of Mice and Antibody Production. Four female BALB/c mice (Forschungsinstitut für Versuchstierzucht, Himberg, Austria) were immunized intraperitoneally starting at the age of 8 wk. For the initial dose, 50–75 μg membrane preparation (29) from E. histolytica strain SFL-3 in 150 μl of 0.9% (wt/vol) NaCl was mixed with 150 μl of complete Freund's adjuvant. On days 27 and 35, animals received the same amount of membrane preparation in incomplete Freund's adjuvant. The antibody response was assayed by ELISA as described below. 3 d before the fusion, mice received an intravenous injection of 100 μl, and 1 d later an injection of 50 μl of amebic membrane antigen in PBS (0.5 μg/μl). 2 d later, the mouse with the highest ELISA titer was killed, and splenocytes were isolated and fused to P3-X-Ag8.653X myeloma cells at a ratio of 5:1 by standard polyethylene glycol method (30, 31). Hybridomas were selected in hypoxanthine-aminopterin-thymidine medium. Hybridomas of interest were subcloned three times by limiting dilution, and grown in hypoxanthine-thymidine (HT) medium supplemented with 10% (vol/vol) fetal calf serum (Sebak, Suben, Austria). Class and subclass of monoclonal antibodies were determined using an ELISA with specific subclass antibodies (PharMingen, San Diego, CA). Small scale protein G purification of antibodies was performed using the MabTrapGII kit (Pharma Biotech AB, Uppsala, Sweden). Fab fragment isolation was done using the ImmunoPureap Fab preparation kit (Pierce Chemical Co., Rockford, IL).

ELISA Methods. ELISA flat-bottomed microplates (Greiner, Kremsmünster, Austria) were coated with 100 μl well of the membrane preparation from E. histolytica SFL-3 (10 μg/ml in 0.1% [wt/vol] ammonium bicarbonate, 0.05% [wt/vol] NaN3) and dried at room temperature overnight. Hybridoma supernatants were added at different dilutions in PBS and incubated at 37°C for 1 h. Plates were washed five times, and bound antibodies were detected with peroxidase-labeled anti-mouse antibodies (Jackson ImmunoResearch Labs., Inc., West Grove, PA) at a 1:1,000 dilution.

Immunoblotting. Amebic membrane antigens were separated by SDS-PAGE with 10% acrylamide gels (32), blotted onto nitrocellulose (Schleicher & Schull, Dassel, Germany) and probed with 1:100 dilutions of hybridoma supernatants in buffer G (50 mM sodium phosphate, 0.5% [vol/vol] Tween 20, 0.5% [wt/vol] bovine serum albumin, and 0.05% [wt/vol] sodium azide, pH 7.5). Bound antibodies were detected with 35S-labeled sheep anti-mouse antibodies (Amersham International, Buckinghamshire, UK).

Metabolic Labeling and Extraction of Crude Glycolipids. Metabolic labeling was performed in a similar way as described by Prasad et al. (19). In brief, SFL-3 trophozoites at a concentration of 2–5 × 10^5/ml of TYI-5-33 glucose- or phosphate-free medium were incubated with either [3H]glucose (0.3 μCi/ml) or [32P]orthophosphate (0.5 μCi/ml) (both DuPont NEN, Cambridge, MA) for 3 h at 36°C. The cells were washed with PBS three times at 4°C and delipidated by extracting with five parts of chloroform/methanol 3:2 and one part of 4 mM MgCl2, next with five parts of chloroform/methanol/water 10:10:3 and one part of chloroform/methanol 1:1, and afterwards with chloroform/methanol water 10:10:3. The crude glycolipids were then extracted with solvent E (water/ethanol/diethylether/pyridine/ammonium hydroxide 15:15:5:1:0.017). The insoluble residue was discarded, and the extract was dried overnight in the vacuum, giving fraction E.

Immunoprecipitation of the Radiolabeled Antibody EH5 Binding Antigen. Crude glycolipids (fraction E) were resuspended in ice-cold RIPA buffer [50 mM Tris/Cl, 150 mM NaCl, 1% [vol/vol] N P-40, 0.5% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] SDS, pH 8.3; reference 30]. Debris was removed by centrifugation, and the supernatant was preadsorbed with protein G-Sepharose (Pharmacia Biotech AB) for 1 h, centrifuged, and protein G-purified antibody EH5, isotype-matched control antibody BIP 1, or an amebiasis patient's serum at a dilution of 1:500 was added to the supernatant and rotated end over end for 2 h at 4°C. Protein G-Sepharose was added for 1 h, and then immune complexes were precipitated by centrifugation, washed at least four times with RIPA buffer, re-suspended in SDS-PAGE loading buffer, and loaded onto a 10% SDS-PAGE gel. [3H]Glucose labeled molecules were visualized by fluorography by treating the gels with EN1HANCE (DuPont NEN) according to the supplier's protocol. Gels containing 32P-
labeled molecules were directly exposed. All gels were exposed to films at −70°C.

Chemical and enzymatic cleavage of the E. histolytica trophozoites was treated with 100 μl of 40 mM trifluoroacetic acid for 8 min at 100°C for mild acid hydrolysis (33). Alternatively, fraction E from 10° E. histolytica SFL-3 trophozoites was treated with 1 U phosphatidylinositol-specific phospholipase C (Pl-PLC) from Balbussus erulus ( Boehminger M annheim G erf, Germany) in 0.1 M Tris/Cl, 0.1% (wt/vol) deoxycholate, pH 7.4, overnight at 37°C. The cleaved preparations or the control without added PI-PLC was extracted with Triton X-114 (34) and the products in the aqueous phase were analyzed (35) by chromatography through phenyl-coupled Sepharose (Sigma Chemical Co., St. Louis, MO). Cleaved samples were diluted in 1.8 ml of 0.1 M acetic acid, 0.1 M NaCl, and loaded onto a phenyl-coupled Sepharose column with 2 ml bed volume. Three fractions of 0.6 ml each were collected. The column was then sequentially washed with 2 × 0.6 ml of 0.1 M acetic acid, 2 × 0.6 ml of H2O, and 6 × 0.6 ml of solvent (see above). All fractions were dried in an atmosphere of nitrogen, resuspended in 10 μl of H2O, and then 1-μl samples were dotted onto nitrocellulose and antigen was detected with antibody EH5 and [35S]-labeled sheep anti–mouse antibodies.

Immunopurification Purification of the E. histolytica Antigen and Interaction with Con A. Protein G-purified antibody EH5 (Eurogentec, Seraing, Belgium) was coupled to 3M Emphaze Biosupport Medium (Pierce Chemical Co.) according to the instructions of the supplier. Fraction E purified from 4 × 107 E. histolytica SFL-3 trophozoites was dissolved in 10 ml of PBS with 0.05% (wt/vol) deoxycholate and loaded on a 2-mL immunopurification column. The column was washed with 20 ml of PBS, and the E. histolytica antigen was eluted with 50 mM diethylamine, pH 11.5. Eluted antigen was column was washed with 20 ml of PBS, and the EH5 antigen was dried in an atmosphere of nitrogen, resuspended in 10 ml of PBS with 0.05% (wt/vol) of nonfat dry milk for 30 min. For in situ epifluorescent labeling of monoclonal antibodies, coverslips were incubated for 30 min in a 1:200 dilution in PBS of rhodamine or FITC-labeled rabbit anti–mouse antibodies (Sigma Chemical Co.), which had been preadsorbed with trophozoites as previously described (37). The preparations were further incubated in PBS containing 1% bovine serum albumin for 30 min at room temperature, briefly washed in PBS, and mounted on a glass slide with 70% (vol/vol) glycerol in PBS.

Confocal Microscopy. Fluorescent samples were examined on a confocal laser scanning microscope (DIAPLAN; Leica, Heidelberg, Germany) equipped with a ×63 objective. Rhodamine-labeled samples were visualized using a high pass R 6505 filter after excitation at 568 nm. Observations were performed in 10 planes from the bottom to the top of each cell. The distance between scanning planes was 0.5 μm. Three dimensional reconstruction of serial confocal sections was performed with the CLSM-Leica software. Photographs were taken on Kodak T-max 400 film (Eastman Kodak Co., Rochester, N.Y.) using a 35-mm camera mounted on a Polaroid Freeze-Frame video monitor.

Transmission Electron Microscopy (TEM) and Immunogold Labeling. Growing trophozoites (108) from E. histolytica strain HM-1:IMSS were harvested by centrifugation at 700 g for 5 min and washed in PBS. Cells were fixed with 4% paraformaldehyde and 0.2% glutaraldehyde in 100 mM Hepes, pH 6.9, for 1 h at 4°C. After one wash with the same buffer, trophozoites were embedded in 10% gelatin and then slowly centrifuged. The pellet was solidified on ice and cut in 1-μm slices which were infused in 1.7 M sucrose and 15% polyvinylpyrrolidone (10,000 mol wt) for varying amounts of time, from 2 h to overnight. The samples were mounted on holder pins and frozen by rapid immersion in liquid nitrogen or in liquid propane. Thin sections were cut at −120°C using a diamond knife on an FCS cryosystem (Reichert, Vienna, Austria). Grids were treated with drops of the following reagents: 50 mM NH4Cl in PBS (10 min), 1% (wt/vol) bovine serum albumin in PBS (5 min), purified antibody EH5, diluted 1:100 in 1% bovine serum albumin in PBS (1 h), PBS (3 washes, 5 min each), anti-mouse IgG + IgM antibodies conjugated with 10-nm gold particles (British Biocell International, Cardiff, UK) diluted in 0.01% (wt/vol) fish skin gelatin (Sigma Chemical Co.) in PBS (30-40 min), PBS (1 min), and distilled water (3 washes, 1 min each). Samples on the grids were then fixed with 1% glutaraldehyde in 100 mM cacodylate, pH 7.4 (2 min), rinsed with distilled water, and embedded in 1% (wt/vol) methylcellulose and 0.3% (wt/vol) uranyl acetate. Sections were observed with an electron microscope (CM12; Philips, Eindhoven, Netherlands) operating at 60 kV.

Passive Immunization of SCID Mice. CB-17 SCID mice (The Jackson Laboratory, Bar Harbor, ME) were treated according to the method of Cieslak et al. (38). Each immunized animal received 200 μl of antibody EH5 (1 mg/ml; n = 12) or isotype-matched control monoclonal antibody HDP-1 (1 mg/ml; n = 6) intraperitoneally 24 h before intraperitoneal challenge. All 18 passively immunized SCID mice, and 7 control SCID mice which received no antibody, underwent direct hepatic inoculation. The animals were fasted for 24 h and subsequently anesthetized by intramuscular application of a combination of ketamine hydrochloride and xylazine. Laparotomy was performed by a vertical incision of ~1 cm to visualize the liver. E. histolytica HM-1:IMSS trophozoites (109) in a volume of 100 μl were injected into the left liver lobe. Peritoneum and abdominal wall were closed by catgut sutures and the skin was closed using clips. After 7 d, animals were killed and the liver was entirely removed, sectioned,
and any abscess detected was resected and weighed separately. The percentage of liver abscessed was calculated as the weight of the abscess divided by the liver weight before abscess removal.

Results

Antibody Production. Using a membrane preparation of E. histolytica SFL-3 as a mixture of antigens, a panel of eight monoclonal antibodies (EH1 to EH8) was raised. All of these antibodies bound to amebic membrane antigens in ELISA and immunoblot assays. This report deals exclusively with antibody EH5 and the EH5 antigen. The EH5 antibody belongs to the IgG1 subclass as determined by ELISA.

Test of Antibodies with Membrane Preparations of Different E. histolytica and E. dispar Strains. Preliminary ELISA tests had shown that antibody EH5 was able to discriminate between E. histolytica and E. dispar (data not shown). Fig. 1 shows the immunoblot results obtained with membrane preparations from different xenically and axenically grown E. histolytica and E. dispar strains using antibody EH5. In addition, a membrane preparation from T. vaginalis, another protozoan parasite which is only distantly related to E. histolytica and E. dispar, was prepared and included. The immunoblots show strong reactivity for all E. histolytica strains of antigens migrating as a polydisperse band, whereas E. dispar bound to antibody EH5 weakly, and T. vaginalis hardly at all. In addition, the signals were stronger when the amebas had been grown axenically.

Characterization of the Antigen Recognized by Antibody EH5. The patterns in the immunoblots resembled patterns that had been previously observed for lipophosphoglycan antigens (19, 20). To test whether the EH5 antigen copurified with lipophosphoglycans, whole E. histolytica SFL-3 trophozoites were delipidated, and glycolipids (fraction E) were extracted. An immunoblot of the fractions demonstrated that most of the EH5 antigen was extracted into fraction E (results not shown). To identify some of the components present in the EH5 antigen, a metabolic labeling experiment was performed. SFL-3 trophozoites were either labeled with [3H]glucose or [32P]orthophosphate. The radiolabeled amebas were washed and delipidated, and fraction E was obtained and resuspended in RIPA buffer. The preparation was preadsorbed with protein G–Sepharose to remove unspecific complexes. The samples were then incubated either with antibody EH5, with isotype-matched control antibody BIP 1 (31), or as a positive control with the serum from an amebiasis patient in a 1:500 dilution. Immune complexes were bound to protein G–Sepharose, precipitated, and analyzed by SDS-PAGE followed by fluorography or autoradiography. Fig. 2 A shows the results for the labeling with [3H]glucose. Only the patient's serum (Fig. 2 A, lane 4) and antibody EH5 (Fig. 2 A, lane 2), but not the control antibody BIP 1 (Fig. 2 A, lane 3), precipitated the antigen. This showed that radiolabeled glucose was incorporated into the EH5 antigen. Fig. 2 B shows the results for the labeling with [32P]orthophosphate. Whole amebas were labeled very heavily (data not shown). Again, antibody EH5 (Fig. 2 B, lane 1) and the patient's serum (Fig. 2 B, lane 2), but not the control antibody BIP 1 (Fig. 2 B, lane 3) precipitated a phosphate-labeled antigen. Thus, EH5 antigen also incorporated radiolabeled phosphate.

To test whether these phosphates were forming acid-labile diester bonds, fraction E from E. histolytica SFL-3 trophozoites was digested for 8 min at 100°C in 40 mM trifluoroacetic acid. The products were analyzed by hydrophobic chromatography on phenyl-coupled Sepharose. The undigested EH5 antigen in fraction E eluted over a large range (Fig. 3, lane B), mild acid digestion reduced the binding of the EH5 antibody, and the partially digested EH5 antigen eluted only in the hydrophobic region (Fig. 3, lane A), in agreement with the degradation of the hydrophilic parts of the molecules.

Figure 1. Immunoblot: membrane preparations from axenically cultured E. histolytica H M-11M SS (1), 200 N IH (2), H K-9 (3), and SFL-3 (4), xenically grown E. histolytica SFL-3 (5), xenically grown E. dispar SAW 760 (6), xenically grown E. dispar SAW 142 (7), and axenically grown T. vaginalis (8) were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with a 1:100 dilution of hamster supernatant from antibody EH5 (A lanes) or buffer only (B lanes). Bound antibodies were detected with 125I-labeled sheep anti-mouse antibodies.

Figure 2. Metabolic labeling experiments. (A) Labeling with [3H]glucose. Lane 1, total PBS washed amebas; lane 2, fraction E, immunoprecipitated with antibody EH5; lane 3, fraction E, immunoprecipitated with isotype-matched control antibody BIP 1; lane 4, fraction E, immunoprecipitated with amebiasis patient's serum. (B) Labeling with [32P]orthophosphate. Lane 1, fraction E immunoprecipitated with antibody EH5; lane 2, fraction E immunoprecipitated with amebiasis patient's serum; lane 3, fraction E immunoprecipitated with control antibody BIP 1. At the left side, molecular masses from a protein marker are given for better orientation. However, these cannot serve as size markers for molecules such as lipophosphoglycans.
Fraction E was also subjected to treatment with PI-PLC, followed by Triton X-114 extraction and hydrophobic chromatography (Fig. 3, lanes C and D). This time, the migration of the EH5 antigen shifted towards a more hydrophilic region, in agreement with the partial cleavage of hydrophobic glycoinositol phospholipid (GPI) membrane anchors from the EH5 antigen. Although we do not yet know the exact chemical structure of the EH5 antigen, the results showed that it could be classified as a lipophosphoglycan.

Binding of C on A to the EH5 antigen and agglutination of trophozoites by the EH5 antibody. Recently, it was shown that components in fraction E from E. histolytica bound to Con A, and therefore were likely to contain terminal mannose residues (23). With a new preparation of protein G-purified EH5 antibody, we were able to immunofinity purify the EH5 antigen as described above. Alkaline elution conditions were used, instead of the more common acidic conditions, to protect the antigen from partial degradation. Fig. 3, lane E, shows dotted fractions from the immunoaffinity column that were probed with antibody EH5. The antigen eluted as a broad peak. In Fig. 3, lane F, the same fractions were dotted onto nitrocellulose, incubated with biotin-labeled Con A, and bound Con A was detected with alkaline phosphatase-labeled streptavidin. Three of the four fractions eluted from the affinity column containing EH5 antigen also bound to Con A, and one of the fractions did not bind. This showed, on the one hand, that the EH5 antigen was chemically heterogeneous, but on the other hand, that a large portion of the EH5 antigen bound to Con A.

If the EH5 antigen, or at least a significant portion of it, was able to bind to Con A, which is known to agglutinate preferentially pathogenic amebas (39, 40), then the EH5 antibody should also be able to agglutinate E. histolytica trophozoites. To test this, E. histolytica SFL-3 trophozoites (2–4 × 10^6/well) were used in an agglutination experiment (Fig. 4) along the lines described for the agglutination by an antibody against a 96-kD antigen (36). Fig. 4A shows the amebas 1 h after addition of only HT hybridoma medium, whereas B shows the results after addition of isotype-matched control antibody BIP 1. Fig. 4C and D shows the amebas 15 and 60 min after addition of antibody EH5 supernatant, whereas E and F show the effect of purified antibody EH5 after 15 and 60 min. The agglutination process began ~15 min after addition of antibody EH5, and after 1 h the amebas were strongly agglutinated. No agglutination was observed when isolated monomeric Fab fragments from antibody EH5 were added (data not shown).

Confocal Immunofluorescence Using Antibody EH5. To determine the cellular localization of the antigen recognized by the EH5 antibody in the amebas by independent means, immunolocalization experiments on the light and electron microscopic levels were performed. First, trophozoites were fixed and incubated with antibody EH5. Two different methods of fixation and permeabilization were used (see Materials and Methods). In method A, amebas were included in the presence of methanol. After labeling, trophozoites were analyzed by laser confocal microscopy (Fig. 5 A) at four different optical planes and revealed a labeling of the entire surface of the amebas. When fixed trophozoites were permeabilized by using Triton X-100 to solubi-
lize the membrane lipids (method B), labeling with antibody EH5 and confocal microscopy (Fig. 5 B) revealed labeling of the periplasmic membrane as well as internal vesicle membranes. Secondary antibodies used alone in control experiments did not label the amebas (results not shown).

**TEM and Immunolabeling.** Cellular localization of the EH5 antigen on the ultrastructural level was examined by TEM and immunogold labeling. Growing trophozoites were fixed and treated for TEM using a protocol specifically adapted for *E. histolytica* (see Materials and Methods) to obtain maximal preservation of antigenic structure. The resulting pictures indicate a localization of the EH5 antigen primarily in association with the membranes. On the plasma membrane (Fig. 6, a and b), gold particles were observed on the outward face, whereas in the internal membrane-bound structures (Fig. 6 a), the gold particles appeared to be localized preferentially on the inner face of the membranes.

**Passive Immunization Study in SCID Mice.** With the immunolocalization studies showing a strong expression of the EH5 antigen on the surface of *E. histolytica*, it was interesting to get some information on whether the antigen might be a possible vaccine candidate. To address this question, we chose the SCID mouse model because it is well-suited for passive immunization studies. Treated mice received a single dose of 200 μg of either isotype-matched control monoclonal antibody HDP-1, antibody EH5, or no treat-
ment 24 h before intrahepatic challenge. 7 d after challenge, the mice were killed and examined for liver abscess formation, and abscess sizes were determined.

All control SCID mice (7 out of 7) and all SCID mice passively immunized with the isotype-matched control monoclonal antibody HDP-1 (6 out of 6) had amebic liver abscesses (Table 1), with a mean abscess size of 16 ± 4% of the liver abscessed in control mice, and 15 ± 6% of the liver abscessed in HDP-1 treated mice. In contrast, only 1 out of 12 mice receiving EH5 monoclonal antibody developed an amebic liver abscess, and the abscess size in this SCID mouse was smaller (9% of the liver abscessed). The difference in the number of mice developing an amebic liver abscess between HDP-1 immunized SCID mice and SCID mice receiving antibody EH5 was highly significant (P < 0.0005).

**Table 1.** Protection of SCID Mice from Amebic Liver Abscess by Passive Immunization with Anti-lipophosphoglycan Monoclonal Antibody EH5

| Vaccine group          | No. of SCID mice with liver abscess/No. of SCID mice challenged | Percentage protected | Percentage of liver abscessed in nonprotected mice mean ± SD |
|------------------------|---------------------------------------------------------------|----------------------|-------------------------------------------------------------|
| Nonimmunized          | 7/7                                                          | 0.0                  | 16 ± 4                                                      |
| HDP-1 (control) immunized | 6/6                                                        | 0.0                  | 15 ± 6                                                      |
| EH5 immunized         | 1/12                                                        | 91.7 (P < 0.0005)*   | 9                                                            |

*Significant difference from control immunized group as determined by Fisher’s exact test.

**Discussion**

In this study, monoclonal antibodies were raised against a membrane preparation from *E. histolytica* to enable us to study surface structures of this parasite, which might be vaccine candidates in the future, and to identify molecules preferentially found on the surface of pathogenic amebas which might be important in the molecular cross-talk with the host.

The monoclonal antibody technology was chosen with the aim of studying defined antigens, and, as an important second criterion, of looking for antibodies with selective binding to *E. histolytica* as compared to *E. dispar*. Antibody EH5 fulfilled these criteria in the best way and was selected for further study. Both immunoblot and ELISA data showed a large difference in the binding of the EH5 antibody between the pathogenic and nonpathogenic species. It remains to be tested if this difference can be exploited for diagnostic purposes.

The polydispersed bands obtained in immunoblots using antibody EH5 resembled those obtained from the previously described monoclonal antibodies 2D7.10, described by Bhattacharya and colleagues (17, 19), and CC 8.6, taken from our laboratory (20). All three antibodies bind to lipophosphoglycan antigens. However, the epitopes recognized seem to be different, because the three antibodies show clear differences in binding to different strains of *E. histolytica*. 2D7.10 binds to an antigen which is almost completely downregulated by the presence of bacteria (21), while EH5 showed much smaller differences in binding between xenic or axenic cultures. The CC 8.6 antibody bound only weakly to strain 200:N1H (20), whereas EH5 exhibited strong binding to 200:N1H (see Fig. 1). Taken together, the expression of certain epitopes of the amebic lipophosphoglycans appears to display strain differences and to be dependent on culture conditions.

The localization of the EH5 antigen was studied by confocal immunofluorescence microscopy and immunogold labeling observed by TEM. The confocal images showed that the labeling on the surface was very intense, but also extended to vesicular structures when stronger permeabilization conditions were applied. The immunogold labeling gave an interpretation to these findings; the EH5 antigen could be directly visualized on the outer face of the plasma membrane and the inner face of what appeared to be a vesicular membrane. Thus, the EH5 lipophosphoglycan antigen forms not only a surface coat, but also appears in the
amebas in inverted vesicles. This demonstrates the difficulty of clearly defining surface localization in this dynamic organism.

The immunoaffinity-purified EH5 antigen bound to Con A in a dot-blot experiment with the exception of one of the antibody-positive fractions from the affinity column. This result pointed again to a significant chemical heterogeneity of the EH5 antigen. More importantly, the preferential agglutination of pathogenic amebas (39, 40) by Con A can be explained at least in part by the binding of Con A to the lipophosphoglycan-like antigen recognized by the EH5 antibody. In agreement with this explanation, the EH5 antibody strongly agglutinated one strain. Antibody EH5 bound to all E. histolytica strains that we were able to test; however, antibody EH5 may fail to be protective against some strains of E. histolytica or in other strains of mice. In the future, it will be very interesting to examine the structure of the EH5 epitope, look for ways to synthesize it in vitro, and test whether such a structure may be a component for active immunization.

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References

1. Walsh, J.A. 1986. Problems in recognition and diagnosis of amebiasis: estimation of the global magnitude of morbidity and mortality. Rev. Infect. Dis. 8:228–238.

2. Diamond, L.S., and C.G. Clark. 1993. A redescription of Entamoeba histolytica Schaudinn, 1903 (emended Walker, 1911) separating it from Entamoeba dispar Brumpt, 1925. J. Eukaryot. Microbiol. 40:340–344.

3. Espinosa-Cantellano, M., and A. Martinez-Palomo. 1991. The plasma membrane of Entamoeba histolytica: structure and dynamics. Biol. Cell. 72:189–200.

4. Kain, K.C., and J.I. Ravdin. 1995. Galactose-specific adhesion mechanisms of Entamoeba histolytica: model for study of enteric pathogens. Methods Enzymol. 253:424–439.

5. Mann, B.J., B.E. Torian, T.S. Vedvick, and W.A. Petri. 1991. Sequence of a cysteine-rich galactose-specific lectin of Entamoeba histolytica. Proc. Natl. Acad. Sci. USA. 88:3248–3252.

6. Tannich, E., F. Ebert, and R.D. Horstmann. 1991. Primary structure of the 170-kDa surface lectin of pathogenic Entamoeba histolytica. Proc. Natl. Acad. Sci. USA. 88:1849–1853.

7. Stanley, S.L., K. Tian, J.P. Koester, and E. Li. 1995. The serine-rich Entamoeba histolytica protein is a phosphorylated membrane protein containing O-linked terminal N-acetylgalactosamine residues. J. Biol. Chem. 270:4121–4126.

8. Braga, L.L., H. Ninomiya, J.J. McCoy, S. Eacker, T. Wiedmer, C. Pham, S. Wood, P.J. Sims, and W.A. Petri. 1992. Inhibition of the complement membrane attack complex by the galactose-specific adhesin of Entamoeba histolytica. J. Clin. Invest. 90:1131–1137.

9. Descoteaux, S., P. Ayala, E. O. Orozco, and J. Samuelson. 1992. Primary sequences of two P-glycoprotein genes of Entamoeba histolytica. Mol. Biochem. Parasitol. 54:201–211.

10. Yi, Y., and J. Samuelson. 1994. Primary structure of the Entamoeba histolytica gene (Ehvma1) encoding the catalytic peptide of a putative vacuolar membrane proton-transporting ATPase (V-ATPase). Mol. Biochem. Parasitol. 66:165–169.
11. Petri, W. A., and J. I. Ravdin. 1991. Protection of gerbils from amebic liver abscess by immunization with the galactose-specific adherence lectin of Entamoeba histolytica. Infect. Immun. 59:97–101.

12. Soong, C. J., K. C. Kain, M. Abd-Alla, T. F. H. G. Jackson, and J. I. Ravdin. 1995. A recombinant cysteine-rich section of the Entamoeba histolytica 170-kD lectin conferring antibody-mediated protection against invasive amebiasis. J. Exp. Med. 185:1793–1801.

13. Lotter, H., T. Zhang, K. B. Seydel, S. L. Stanley, and E. Tan- nich. 1997. Identification of an epitope on the Entamoeba histolytica 170-kD lectin conferring antibody-mediated protection against invasive amebiasis. J. Exp. Med. 185:1793–1801.

14. Zhang, T., P. R. Cieslak, and S. L. Stanley. 1994. Protection of gerbils from amebic liver abscess with a recombinant Entamoeba histolytica antigen. Infect. Immun. 62:1166–1170.

15. Soong, C. J., B. E. Torian, M. D. Abd-Alla, T. F. H. G. Jackson, V. Gatharim, and J. I. Ravdin. 1995. Protection of gerbils from amebic liver abscess by immunization with recombinant Entamoeba histolytica 29-kilodalton antigen. Infect. Immun. 63: 472–477.

16. Isibasi, A., M. Santa Cruz, A. Ramirez, and J. Kumate. 1982. Immunomimicry of a lipopeptidolipofucosanica extrada de trofozoitos de Entamoeba histolytica cepa HK-9 cultivados en medio axenico. Utilizando el metodo de fenol-agua. An. Inst. V. 13:51–55.

17. Bhattacharya, A., R. Ghidyal, S. Bhattacharya, and L. S. Diamond. 1990. Characterization of a monoclonal antibody that selectively recognizes a subset of Entamoeba histolytica isolates. Infect. Immun. 58:3458–3461.

18. Bhattacharya, A., R. Prasad, and D. L. Sacks. 1992. Identification and partial characterization of a lipophosphoglycan from a pathogenic strain of Entamoeba histolytica. Mol. Biochem. Parasitol. 56:161–168.

19. Prasad, R., M. Tola, S. Bhattacharya, M. P. Sharma, and A. Bhattacharya. 1992. Recognition of Entamoeba histolytica lipophosphoglycan by a strain-specific monoclonal antibody and human immune serum. Mol. Biochem. Parasitol. 56:279–288.

20. Stanley, S. L., H. Huizenga, and E. Li. 1992. Isolation and partial characterization of a surface glycoconjugate of Entamoeba histolytica. Mol. Biochem. Parasitol. 50:127–138.

21. Bhattacharya, A., R. Ghidyal, J. Prasad, S. Bhattacharya, and L. S. Diamond. 1992. Modulation of a surface antigen of Entamoeba histolytica in response to bacteria. Infect. Immun. 60: 1711–1713.

22. Srivastava, G., M. T. Anand, S. Bhattacharya, and A. Bhattacharya. 1995. Lipophosphoglycan is present in distinctly different form in different Entamoeba histolytica strains and absent in Entamoeba moshkovskii and Entamoeba invadens. J. Eukaryot. Microbiol. 42:617–622.

23. Moody, S., S. Becker, Y. Uchamowitz, and D. Mirelman. 1997. Virulent and avirulent Entamoeba histolytica and E. dispar differ in their cell surface phosphorylated glycolipids. Parasitology. 114:95–114.

24. Diamond, L. S., D. R. Harlow, and C. C. Cunnick. 1978. A new medium for the axenic cultivation of Entamoeba histolytica and other Entamoeba. Trans. R. Soc. Trop. Med. Hyg. 72: 431–432.

25. Clark, C. G. 1995. Axenic cultivation of Entamoeba dispar Brumpt 1925, Entamoeba insolita Geiman and Wichterman 1937 and Entamoeba ranarum Grasse 1879. J. Eukaryot. Microbiol. 42:590–593.

26. Diamond, L. S. 1982. A new liquid medium for xenic cultivation of Entamoeba histolytica and other lumen-dwelling protozoa. J. Parasitol. 68:958–959.

27. Robinson, G. L. 1968. The laboratory diagnosis of human parasitic amoebae. Trans. R. Soc. Trop. Med. Hyg. 62:285–294.

28. Diamond, L. S. 1957. The establishment of various trichomonads of animals and man in axenic culture. J. Parasitol. 43:488–490.

29. Ramwani, J., and R. K. Mishra. 1986. Purification of bovine striatal dopamine D-2 receptor by affinity chromatography. J. Biol. Chem. 261:8894–8898.

30. Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 726 pp.

31. Jarolim, E., M. Tejkl, M. Rohac, G. Schlerka, O. Scheiner, D. Kraft, M. Breitenbach, and H. Rumpold. 1989. Monoclonal antibodies against birch pollen allergens: characterization by immunoblotting and use for single-step affinity purification of the major allergen Bet v 1. Int. Arch. Allergy Appl. Immunol. 90:54–60.

32. Fling, S. P., and D. S. Gregerson. 1986. Peptide and protein molecular weight determination by electrophoresis using a high-molarity Tris buffer system without urea. Anal. Biochem. 155:83–88.

33. McConville, M. J., L. E. Thomas-Oates, M. A. Ferguson, and S. Homans. 1990. Structure of the lipophosphoglycan from Leshmania major. J. Biol. Chem. 265:19611–19623.

34. Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. 256:1604–1607.

35. Turco, S. J., and D. L. Sacks. 1991. Expression of a stage-specific lipophosphoglycan in Leshmania major amastigotes. Mol. Biochem. Parasitol. 45:91–99.

36. Torian, B. E., S. A. Lukehart, and W. E. Stamm. 1987. Use of monoclonal antibodies to identify, characterize, and purify a 96,000-Dalton surface antigen of pathogenic Entamoeba histolytica. J. Infect. Dis. 156:343–343.

37. Rahim, Z., A. Raymond-Denise, P. Sansonetti, and N. Guillen. 1993. Localization of myosin heavy chain A in the human pathogen Entamoeba histolytica. Infect. Immun. 61:1048–1054.

38. Cieslak, P. R., H. W. Virgin, and S. L. Stanley. 1992. A severe combined immunodeficient (SCID) mouse model for infection with Entamoeba histolytica. J. Exp. Med. 176:1605–1609.

39. Martinez-Palomo, A., A. Gonzalez-Robles, and M. de la Torre. 1973. Selective agglutination of pathogenic strains of Entamoeba histolytica induced Con A. N. at. N ew Biol. 245:186–187.

40. Trissl, D., A. Martinez-Palomo, C. Arguello, M. de la Torre, and R. de la Hoz. 1977. Surface properties related to concanavalin A-induced agglutination. A comparative study of several Entamoeba strains. J. Exp. Med. 145:652–665.