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 [^{32}P]phosphate or [^{3}H]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-Cantellano 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

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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 immunoaffinity-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-S-33 with *Crithidia fasciculata*, and for the experiments the *Crithidiae* were omitted. The *E. dispar* strain SAW142 was cultured xenically in TYSGM-9 medium (26). *E. histolytica* strain SFL-3 was also cultured xenically in 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.

Membrane Antigen Preparation. Membrane antigens were prepared as described by Ramwani 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 centrifugation at 700 *g* for 10 min at 4°C and discarded. 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)[1] 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 antisubclass antibodies (PharMingen, San Diego, CA). Small scale protein G purification of antibodies was performed using the Mab Trap GII kit (Pharmacia Biotech AB, Uppsala, Sweden). F_{ab} fragment isolation was done using the ImmunoPure® F_{ab} 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] NaN_3) 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 horseradish 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 & Schüll, 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 ^{125}I-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 × 10^6/ml of TYI-S-33 glucose- or phosphate-free medium were incubated with either [^{3}H]glucose (0.3 mCi/ml) or [^{32}P]orthophosphate (0.5 mCi/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 $MgCl_2$, 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] NP-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, resuspended in SDS-PAGE loading buffer, and loaded onto a 10% SDS-PAGE gel. [^{3}H]Glucose labeled molecules were visualized by fluorography by treating the gels with EN^{3}HANCE (DuPont NEN) according to the supplier's protocol. Gels containing ^{32}P-

[1] *Abbreviations used in this paper:* HT, hypoxanthine-thymidine; PI-PLC, phosphatidylinositol-specific phospholipase C; TEM, transmission electron microscopy.

labeled molecules were directly exposed. All gels were exposed to films at −70°C.

Chemical and Enzymatic Cleavage of the EH5 Antigen, and Analysis by Hydrophobic Chromatography. Isolated fraction E from 10^7 *E. histolytica* SFL-3 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 10^7 trophozoites was treated with 1 U phosphatidylinositol-specific phospholipase C (PI-PLC)[1] from *Bacillus cereus* (Boehringer Mannheim, Mannheim, 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 H_2O, and 6 × 0.6 ml of solvent E (see above). All fractions were dried in an atmosphere of nitrogen, resuspended in 10 μl of H_2O, and then 1-μl samples were dotted onto nitrocellulose and antigen was detected with antibody EH5 and ^{125}I-labeled sheep anti–mouse antibodies.

Immunoaffinity Purification of the EH5 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 × 10^7 *E. histolytica* SFL-3 trophozoites was dissolved in 10 ml of PBS with 0.05% (wt/vol) deoxycholate and loaded on a 2-ml immunoaffinity column. The column was washed with 20 ml of PBS, and the EH5 antigen was eluted with 50 mM diethylamine, pH 11.5. Eluted antigen was immediately transferred into PBS buffer using Microcon columns (Amicon Inc., Beverly, MA). 1 μl of each fraction was dotted onto nitrocellulose strips, and bound EH5 antigen was detected with antibody EH5, which was visualized with alkaline phosphatase–coupled secondary antibodies. For the Con A binding experiment, the strips were incubated with biotin-labeled Con A (1:200 in buffer G, see above), and bound Con A was detected with alkaline phosphatase–labeled streptavidin (1:500 in buffer G).

Agglutination Test. The test was performed in a similar way to that described by Torian et al. (36). *E. histolytica* SFL-3 trophozoites (2–4 × 10^4/well) were added to 96-well flat-bottomed microtiter plates. Hybridoma supernatants of antibody EH5, protein G–purified antibody EH5, F_{ab} fragments from antibody EH5, isotype-matched negative control antibody BIP 1 directed against the birch pollen allergen Bet v 1 (31), or HT medium as a control was added to the wells. Plates were further incubated at 37°C. Photographs were taken before and 15 and 60 min after the addition of antibodies and controls.

Indirect Immunofluorescence of E. histolytica. Growing HM-1:IMSS trophozoites were washed with PBS at 37°C, transferred to a coverslip in a 24-mm petri dish, and incubated at 37°C for 10 min to promote attachment. PBS was removed and coverslips were incubated in 3.7% (wt/vol) paraformaldehyde for 15 min at room temperature. One part of the samples was placed in methanol at −20°C for 3 min (treatment A), and the rest were treated by 0.25% (vol/vol) of Triton X-100 in PBS for 3 min (treatment B). Coverslips were washed with PBS, incubated in the presence of 50 mM NH_4Cl for 30 min, and then in PBS containing 1% (wt/vol) of nonfat dry milk for 30 min. For in situ epifluorescent labeling of monoclonal antibodies, coverslips were incubated with 1:100 diluted monoclonal antibodies (purified over protein G column) for 1 h. After two washes with PBS, samples 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 R6590 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, NY) using a 35-mm camera mounted on a Polaroid Freeze-Frame video monitor.

Transmission Electron Microscopy (TEM) and Immunogold Labeling. Growing trophozoites (10^7) 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-mm^3 pieces 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 NH_4Cl 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 intrahepatic 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 (10^6) 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 *Trichomonas vaginalis*, another protist 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 [^{3}H]glucose or [^{32}P]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 [^{3}H]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 [^{32}P]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.

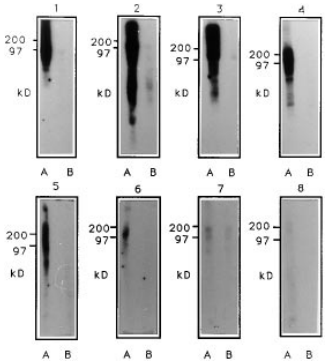

Figure 1. Immunoblot: membrane preparations from axenically cultured *E. histolytica* HM-1:IMSS (*1*), 200:NIH (*2*), HK-9 (*3*), and SFL-3 (*4*), xenically grown *E. histolytica* SFL-3 (*5*), axenically grown *E. dispar* SAW760 (*6*), xenically grown *E. dispar* SAW142 (*7*), and axenically grown *T. vaginalis* (*8*) were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with a 1:100 dilution of hybridoma supernatant from antibody EH5 (*A* lanes) or buffer only (*B* lanes). Bound antibodies were detected with ^{125}I-labeled sheep anti–mouse antibodies.

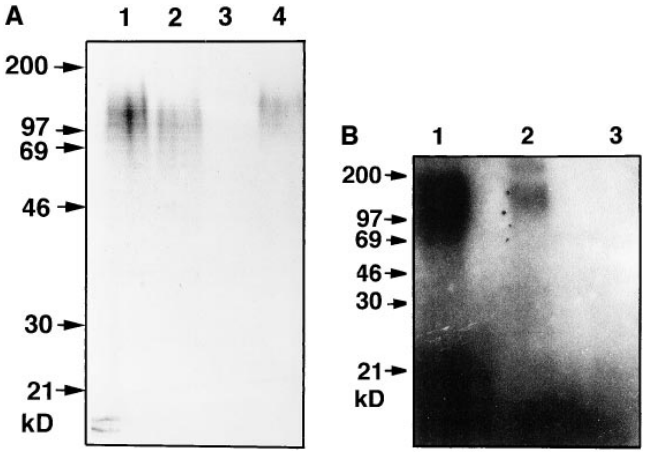

Figure 2. Metabolic labeling experiments. (A) Labeling with [^{3}H]glucose. Lane *1*, total PBS washed amebas; lane *2*, fraction E, immunoprecipitated with antibody EH5; lane *3*, fraction E, immunoprecipitated with isotype-matched control antibody BIP 1; lane *4*, fraction E, immunoprecipitated with amebiasis patient's serum. (*B*) Labeling with [^{32}P]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.

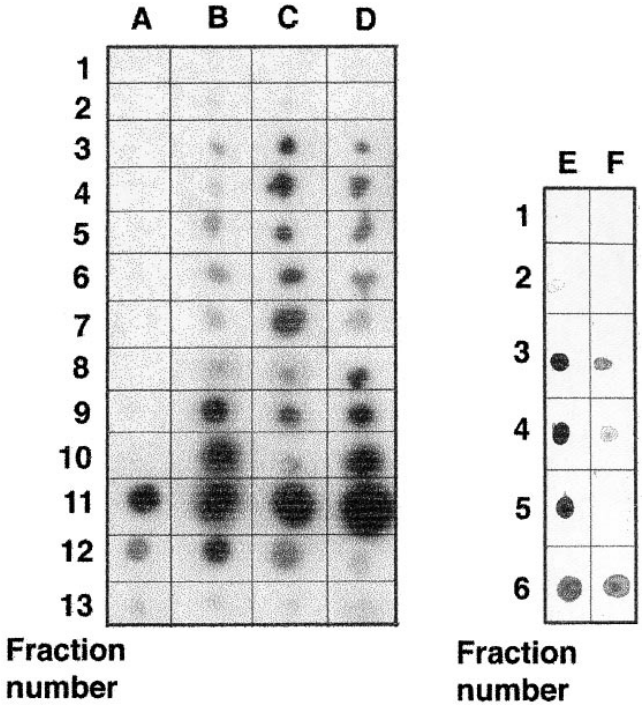

Figure 3. Sensitivity of the EH5 antigen towards mild acid digestion (lanes *A* and *B*), and towards PI-PLC (lanes *C* and *D*) and binding to Con A (lanes *E* and *F*). For lanes *A–D*, fractions were chromatographed through phenyl-coupled Sepharose, eluted fractions were dotted onto nitrocellulose and reacted with antibody EH5, and bound antibody was detected with iodine-labeled sheep anti–mouse antibodies; lane *A*, mild acid digestion; lane *B*, undigested control; lane *C*, PI-PLC digestion; lane *D*, control incubated in PI-PLC buffer only. For lanes *E* and *F*, eluted fractions from immunoaffinity column (*E*) tested with antibody EH5, bound antibodies detected with alkaline phosphatase–coupled anti–mouse antibodies; (*F*) tested with biotin-labeled Con A, bound Con A detected with alkaline phosphatase–coupled streptavidin.

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 Con 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 immunoaffinity 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 and 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.

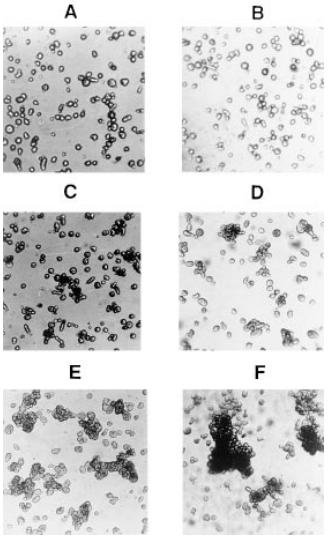

Figure 4. Agglutination test: *E. histolytica* trophozoites in HT medium after 1 h of incubation (*A*); with isotype-matched control antibody BIP 1 after 1 h of incubation (*B*); with supernatant of antibody EH5 after 15 min of incubation (*C*); the same after 1 h of incubation (*D*); with protein G–purified antibody EH5 after 15 min of incubation (*E*); and the same after 1 h of incubation (*F*).

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 ($2–4 \times 10^4$/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. 4 *A* 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. 4, *C* 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 F_{ab} 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 incubated 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-

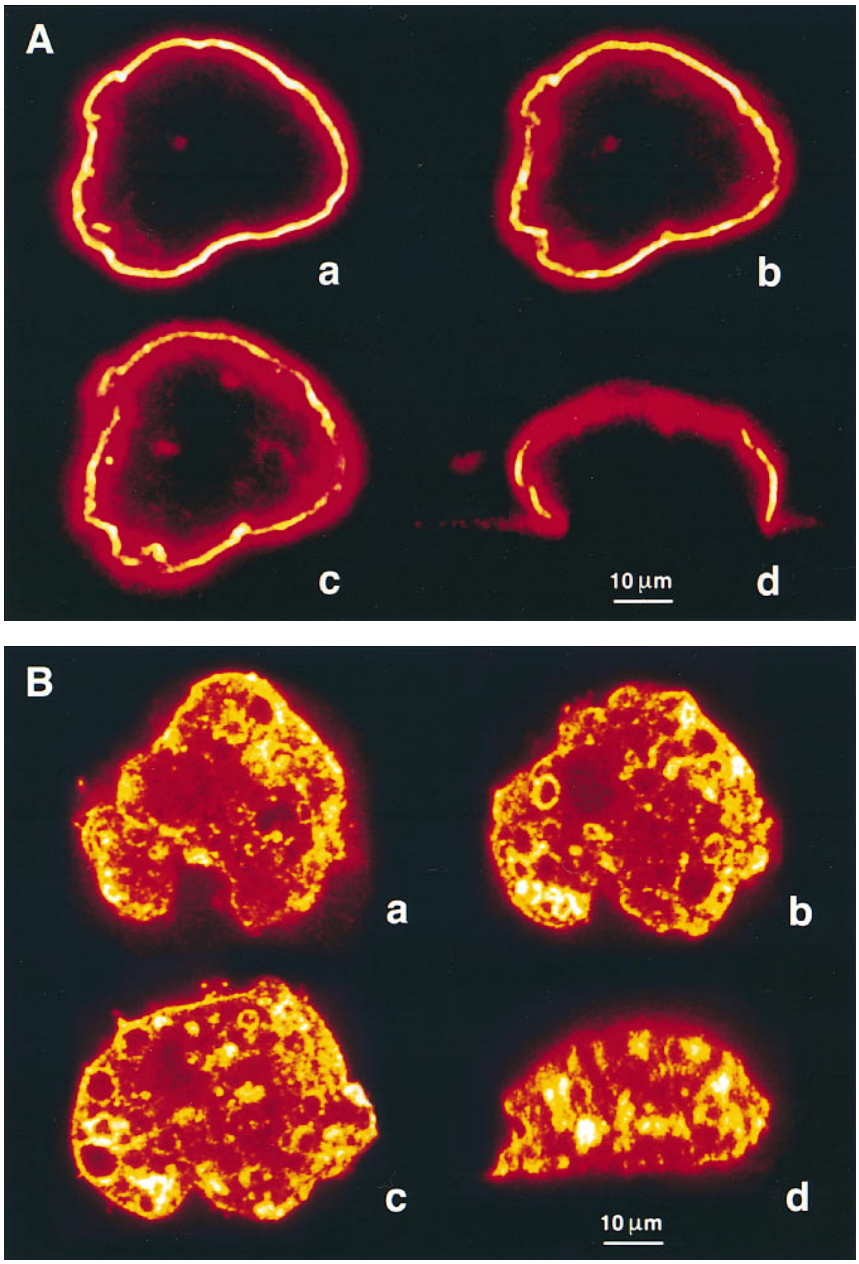

Figure 5. Localization of the antigen binding to antibody EH5 in *E. histolytica* trophozoites by indirect immunofluorescence using confocal microscopy. (*A*) Amebas treated with methanol; (*B*) amebas treated with Triton X-100 for stronger permeabilization. Planes *a*, *b*, and *c* are horizontal planes, and plane *d* is a vertical plane through the middle of the ameba.

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-

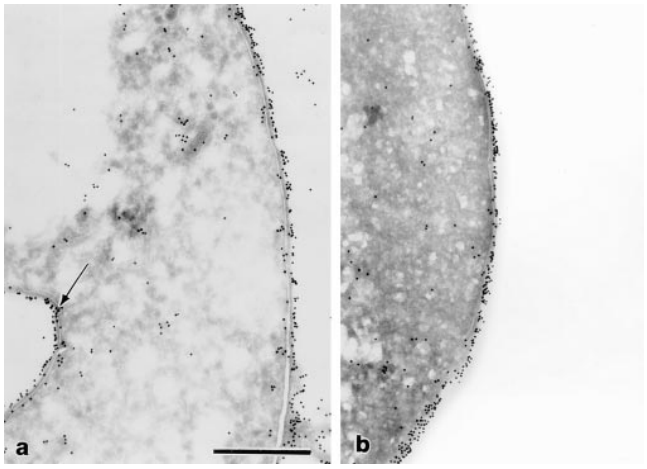

Figure 6. Cryosection immunogold staining of trophozoites stained with antibody EH5, showing two areas of the trophozoite with (*a*) or without (*b*) internal membrane-bound vesicle (bar = 0.5 μm).

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 ± 7% 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 ($P < 0.0005$).

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 polydisperse 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:NIH (20), whereas EH5 exhibited strong binding to 200:NIH (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

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.

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 *E. histolytica* trophozoites. However, it is clear that other structures such as glycoproteins on the amebal surface are also likely to play a role in the agglutination by Con A. Also, it is not known if Con A bound to a lipophosphoglycan is capped by the amebas.

Recently, it was shown, with polyclonal antibodies to defined regions of the galactose- and *N*-acetylgalactosamine–inhibitable lectin, that, depending on the epitopes recognized, passive immunization can either protect SCID mice from amebic liver abscess or it can exacerbate the disease (13). Passive immunization with the lipophosphoglycan-specific antibody EH5 conferred highly significant protection against amebic liver abscess. This is the first report on a single monoclonal antibody with this ability, and the first report relating protection to a glycan epitope.

Significantly, the SFL-3 strain, against which the antibodies were raised, and the HM-1:IMSS challenge strain were different, so the protection is not strictly limited to 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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