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Differentiation of Pathogenic *Entamoeba histolytica* Infections from Nonpathogenic Infections by Detection of Galactose-Inhibitable Adherence Protein Antigen in Sera and Feces

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We determined whether epitope-specific monoclonal antibodies to the galactose-inhibitable adherence protein (GIAP) of *Entamoeba histolytica* could be used in an enzyme-linked immunosorbent assay (ELISA) to detect antigen in serum and feces and differentiate between nonpathogenic zymodemes and the potentially invasive pathogenic organisms that require treatment. Overall, 57% of subjects from Cairo, Egypt, with symptomatic intestinal amebiasis and 42% with asymptomatic infection possessed GIAP antigen in their sera, whereas 4% of uninfected controls or subjects with other parasitic infections possessed GIAP antigen in their sera ($P < 0.001$). In subjects from Durban, South Africa, only 6% of uninfected controls or those with nonpathogenic *E. histolytica* infection were positive for GIAP in serum, whereas 3 of 4 with asymptomatic pathogenic intestinal infection and 75% with amebic liver abscess were positive for GIAP in serum. Fifteen stool samples from patients with intestinal amebiasis were available for study; all had a positive ELISA result for fecal GIAP antigen. Epitope-specific monoclonal antibodies identified 8 of 15 subjects with fecal antigen from pathogenic strains. Seven of those eight subjects had adherence protein antigen in their sera, whereas none of seven with apparent nonpathogenic *E. histolytica* infection had adherence protein antigen in their sera. In summary, we were able to detect *E. histolytica* adherence protein antigen directly in serum and fecal samples by ELISA. The presence of amebic antigen in serum demonstrated 94% specificity for pathogenic *E. histolytica* infection, and amebic antigen is present during asymptomatic intestinal infection. In conjunction with antibody detection, this method should be very useful in the diagnosis and management of intestinal amebiasis.

Entamoeba histolytica is an enteric protozoan that infects 10% of the world's population, especially those in developing countries (28). Pathogenic and nonpathogenic strains have been defined by isoenzyme analysis of cultured isolates (23), epidemiologic studies (7), antigenic differences (17), and distinctive genomic DNA (6, 24, 25). Depending on the site of study, 44 to 90% of asymptomatic infections are caused by nonpathogenic organisms (2), which are not associated with an antiamebic antibody response in serum (12, 21). A minority of subjects with pathogenic *E. histolytica* infections of the intestines present with symptomatic amebic colitis or liver abscess, yet all develop an antiamebic antibody response in serum (12, 21). Currently, there are no widely available diagnostic tests that rapidly detect intestinal *E. histolytica* infection or differentiate pathogenic from nonpathogenic organisms. Diagnosis of infection is dependent on expert microscopic examination of fecal samples and detection of antiamebic antibodies in serum. Diagnosis by microscopy is frequently erroneous, and antibody detection is of reduced utility in areas endemic for *E. histolytica*, where 25% or more of the population is seropositive.

Recently, a number of *E. histolytica* surface antigens have been well characterized. Among these is the galactose-inhibitable adherence protein (GIAP), which mediates amebic attachment to colonic mucins, epithelial cells, and host inflammatory cells and whose binding is necessary for amebic lysis of mammalian target cells (16, 18). We developed an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies to the 170-kDa heavy subunit of GIAP (16, 17, 19, 22) to detect this antigen in serum and feces. These monoclonal antibodies were characterized by Petri et al. (17) to recognize epitopes specific to pathogenic or nonpathogenic strains. We wanted to determine whether GIAP antigen could be detected in serum and feces and whether this effectively differentiated infections caused by pathogenic *E. histolytica* strains from those caused by nonpathogenic strains.

MATERIALS AND METHODS

Study populations. The sera available for study of GIAP antigen included samples from 50 healthy controls in the United States, 50 healthy Egyptian controls without intestinal amebiasis (negative microscopy), 50 individuals from Egypt who were asymptomatic passers of cysts, and 100 Egyptians with symptomatic amebic colitis who presented with bloody diarrhea, hematophagous trophozoites in their stools, a negative stool culture for *Shigella* species, and a positive clinical response to specific antiamebic therapy with metronidazole. Additional control sera included samples from 20 Egyptian patients with bilharzial colitis (dysentery and *Schistosoma mansoni* ova only in stools), 22 Egyptians with non-*Entamoeba* intestinal parasites, 21 controls from the United States with biopsy-confirmed idiopathic inflammatory bowel disease, and 12 recently evaluated subjects in

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South Africa with amebic liver abscess. In addition, studies were performed on sera from South African subjects which were previously studied for serum anti-GIAP immunoglobulin G antibodies. These subjects were identified by prospective screening, and stool cultures were done and zymodeme analyses were performed. This included 35 controls with negative stool cultures, 34 subjects with asymptomatic nonpathogenic infections, and 4 individuals with asymptomatic pathogenic infections.

ELISA for the detection of GIAP antigen in feces was done on 15 samples from Egyptian patients with symptomatic amebic colitis and 26 samples from patients with no evidence of amebic disease admitted to the Cleveland Veterans Affairs Medical Center. Feces from patients and controls were identically stored by freezing at −70°C without any fixative until they were studied.

Production of anti-GIAP antigen monoclonal antibodies. The production and epitope mapping of murine anti-GIAP monoclonal antibodies were reported by Petri et al. (17). The designations and epitope specificities of the monoclonal antibodies used in the present study are as follows: 3F4 to epitope 1, 8A3 to epitope 2, 8C12 to epitope 4, and 1G7 to epitope 5 (17). Antibodies were purified from ascites by precipitation in 50% ammonium sulfate fraction and protein A affinity chromatography or preparative isoelectric focusing. In the present study, two monoclonal antibody systems were used for ELISA. The first detected the GIAP antigens of both pathogenic and nonpathogenic *E. histolytica* isolates by having 3F4 as a capture antibody and 8A3 as a secondary antibody. The second system exclusively detected GIAP antigen from pathogenic *E. histolytica* (17), with 8C12 used as a capture antibody and 1G7 used as a secondary antibody.

Conjugation of secondary antibodies to alkaline phosphatase was done as described previously (1, 4). Briefly, 35 μl of alkaline phosphatase (3 mg; Boehringer Mannheim) was mixed with 120 μl of potassium phosphate buffer (50 mM; pH 7.2) and 1 μl of glutaraldehyde; purified antibody in potassium phosphate buffer was added, and the mixture was incubated for 70 min at room temperature. Following overnight dialysis at 4°C in 0.05 M phosphate-buffered saline (PBS; pH 7.2), the dialysate was mixed with 0.7 ml of Tris-HCl buffer (50 mM; pH 8.0) containing NaCl (0.1 M), $MgCl_2$ (1 mM), and sodium azide (0.1%; wt/vol). Bovine serum albumin (BSA) at 10 μg/ml was added, and the antibody preparation was stored at 4°C until it was used.

ELISA for detection of GIAP antigen in serum and feces. Microtiter plates (96-well, flat-bottom polystyrene ELISA plates; Coster, Cambridge, Mass.) were used. Wells were coated with monoclonal antibody 3F4 or 8C12 (1.6 μg per well) in 100 μl of coating buffer (80 ml of 0.2 M Na_2CO_3 and 170 ml of 0.2 M $NaHCO_3$) by incubation overnight at 4°C. Following washing with PBS-Tween (pH 7.5), nonreactive sites were blocked with 1% BSA. Serum or feces was added to each well for overnight incubation at 4°C. Sera were diluted 1:100 in PBS-Tween with 1% BSA; feces were mixed with an equal volume of PBS (pH 7.4) containing 2 mM phenylmethylsulfonyl fluoride. The wells were washed extensively and the alkaline phosphatase-conjugated 8A3 or 1G7 antibodies (0.5 μg/ml) were added (1 μl of a 1:1,000 dilution) in PBS-Tween with 1% BSA for incubation at room temperature for 2 h. Wells were washed, and developing solution (1 M diethanolamine, 1 mM $MgCl_2$ [pH 9.8]) with *p*-nitrophenyl phosphate disodium (1 mg/ml) was added. The reaction was developed in the dark for 2 h, and the degree of color changes (optical density at 405 nm) was measured in an ELISA plate reader. Results were corrected for nonspecific background by subtracting the optical density of test wells from that of the paired wells not coated with primary antibody but otherwise subject to the identical procedure. Each serum and fecal sample was studied in duplicate.

PEG precipitation of GIAP-containing immune complexes in serum. Immune complexes were isolated from human serum (16, 17) by mixing 20 μl of serum with 200 μl of 0.2 M disodium EDTA (pH 7.5) and 100 μl of 12% polyethylene glycol (PEG) in 0.1 M borate buffer (pH 8.3). The mixture was incubated for 18 h at 4°C and was centrifuged at 6,000 rpm (Beckman Ultracentrifuge) for 30 min. The supernatant was discarded and the precipitate was washed with 500 μl of cold 2.4% PEG. The precipitated immune complexes were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with anti-GIAP monoclonal antibodies.

PEG-precipitated immune complexes were boiled in 10 μl of SDS-PAGE sample buffer and were electrophoresed on 8% separating gels and a 4% stacking gel formed from 30% acrylamide and 0.8% *N,N*-methylenebisacrylamide (3, 14). Gels were soaked in transfer buffer (0.025 M Tris, 187 mM glycine, 10% methanol) for 30 min and were electrophoretically transferred to nitrocellulose with a transblot apparatus (Bio-Rad). The nitrocellulose membrane was blocked by exposure to 4 mM Tris-HCl and 150 M NaCl (pH 7.5) containing 5% BSA. The presence of GIAP antigen was demonstrated by immunoblotting with a mixture of monoclonal antibodies 3F4, 8C12, 8A3, 1G7, and 5B8 (0.01 mg/ml) and an anti-mouse horseradish peroxidase-conjugated secondary antibody. The reaction was developed by incubation with 30 μl of H_2O_2 in 50 ml of 50 mM Tris-HCl–100 mM NaCl–50 mM $MgCl_2$ with 16.8 mM 4-chloronaphthol in methanol.

Statistics. Significance of difference was determined by Student's *t* test for paired or unpaired data or the Fisher exact test, as appropriate.

RESULTS

Detection of *E. histolytica* GIAP antigen in serum. Levels of GIAP antigen in the sera of all Egyptian study group subjects and U.S. control subjects are indicated by optical density values in Fig. 1. The monoclonal antibodies used in the ELISA detected in serum GIAP derived from either pathogenic or nonpathogenic *E. histolytica*. A positive value was determined as two standard deviations above the mean for the Egyptian control population. We found that 42% of Egyptian subjects with asymptomatic *E. histolytica* infection and 57% with amebic colitis had GIAP antigen in their sera. For comparison, sera from only 2 of 50 U.S. controls (4%), 8% of healthy Egyptian controls, 1 of 22 Egyptians infected with other enteric parasites, 4 of 20 Egyptians (20%) with bilharzial colitis, and 1 of 21 U.S. subjects with idiopathic inflammatory bowel disease were positive in the ELISA for GIAP antigen in serum ($P < 0.01$ for all control groups compared with either group of infected subjects). Stool cultures were not done and zymodeme analyses were not performed for the Egyptian subjects.

We studied a number of sera from South African subjects who had well-characterized fecal isolates (21). Again, ELISA positivity for GIAP antigen in serum was defined as 2 standard deviations above the mean for 35 culture-negative controls from the identical geographic region. Only 2 of 34 individuals with asymptomatic nonpathogenic *E. histolytica* infections were found to have GIAP antigen in their sera (identical to the control group; Fig. 2). In contrast, three of

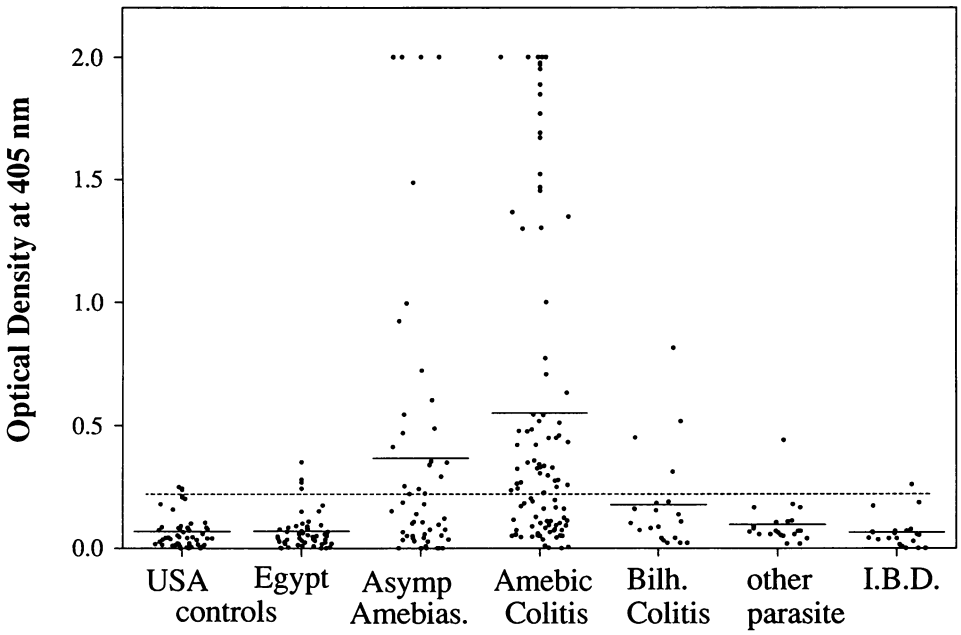

FIG. 1. Detection of *E. histolytica* GIAP antigen in sera by ELISA in subjects with intestinal amebiasis. Study groups are described in detail in the text. I.B.D., inflammatory bowel disease; Bilh. colitis, bilharzial colitis. Sera were from subjects in Egypt and from controls and subjects with inflammatory bowel disease in the United States. A cutoff of 2 standard deviations above the mean for Egyptian controls (optical density, 0.213) was used to define a positive ELISA result. Forty-two percent of subjects with asymptomatic infection (Asymp Amebias) and 57% of subjects with symptomatic colitis had GIAP antigen in their sera ($P < 0.05$ for each group of subjects compared with all control groups).

four serum specimens from subjects with asymptomatic pathogenic intestinal infection possessed GIAP antigen (Fig. 2). Amebic liver abscess results exclusively from infection by pathogenic *E. histolytica*; 75% of subjects with amebic liver abscess were found to have GIAP antigen in their sera (Fig. 2). All of the positive serum samples were obtained during an acute presentation with right upper quadrant pain and fever. The sensitivity of serum antigen detection for the identification of pathogenic *E. histolytica* infection was 68.75%. The specificity was higher, at 94.2%, providing a positive predictive value of 0.733 and, importantly, a negative predictive value of 0.071.

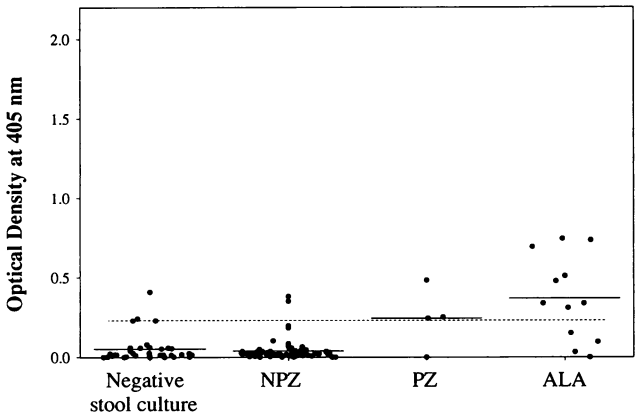

FIG. 2. Detection of GIAP antigen in sera of subjects with asymptomatic *E. histolytica* intestinal infection or with amebic liver abscess (ALA). NPZ, nonpathogenic zymodeme infection; PZ, pathogenic zymodeme infection. Note that only 2 of 35 subjects with negative cultures and 2 of 34 subjects with nonpathogenic intestinal zymodeme infections had GIAP antigen in their sera, in comparison with three of four subjects with intestinal pathogenic zymodeme infections and 8 of 12 subjects with amebic liver abscesses.

Characterization of GIAP antigen in serum. To confirm the specificity of the ELISA for the heavy subunit of GIAP and to further characterize the form of the antigen present in sera, we immunoblotted PEG-precipitated serum immune complexes with the anti-GIAP monoclonal antibodies. We found a discrete immunoreactive protein with a slightly altered mobility in SDS-PAGE (150 kDa) compared with that of the native 170-kDa GIAP heavy-subunit protein purified from axenic trophozoites (Fig. 3). GIAP was demonstrated to be present in sera from subjects with colitis and absent from sera from controls (Fig. 3).

Detection of *E. histolytica* GIAP antigen in feces with correlation to studies of sera. Fifteen stool samples from Egyptian subjects with amebic colitis were available for study and were compared with samples from 26 U.S. subjects with no evidence of amebic disease. By defining a positive ELISA result as the mean plus 3 standard deviations above the mean for U.S. controls, all 15 Egyptian patients with colitis were found to have GIAP antigen in their feces, whereas only 1 of 26 controls were found to have GIAP antigen in their feces ($P < 0.01$) (Fig. 4). The monoclonal antibodies used in this ELISA (3F4 and 8A3) can detect GIAP antigen from both pathogenic and nonpathogenic *E. histolytica* isolates (17).

A correlation of the findings in serum and feces in these patients further demonstrated that GIAP antigenemia in serum occurs predominantly during pathogenic *E. histolytica* infection. Studying two different monoclonal antibody systems (3F4 and 8A3 in comparison with 8C12 and 1G7) to differentiate in feces the GIAP heavy subunit derived from pathogenic and nonpathogenic *E. histolytica*, we found that

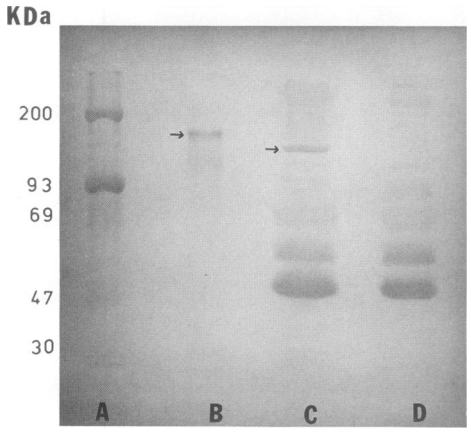

FIG. 3. Immunoblot of sera with anti-GIAP monoclonal antibodies. Lane A, molecular size standards; lane B, immunoaffinity-purified GIAP with the 170-kDa heavy subunit (arrow) recognized by a combination of monoclonal antibodies (3F4, 8C12, 8A3, 1G7, and 5B8); lane C, immunoreactive heavy subunit present in PEG-precipitated serum immune complexes from a patient with amebic liver abscess migrating at 150 kDa (arrow); compare lane C with lane D, which contains immune complexes from sera of control subjects without any evidence of the heavy subunit on immunoblotting.

TABLE 1. Detection of GIAP in feces and sera of 15 patients with intestinal amebiasis

Patient no.	OD value by ELISA[a]		
	Feces PZ + NPZ specific	Feces PZ specific	Serum PZ + NPZ specific
1	0.353	0.00	0.05
2	0.435	0.663	0.420
3	0.752	1.241	0.275
4	0.525	0.242	0.323
5	2.00	0.342	0.329
6	0.962	0.121	0.003
7	0.547	1.085	1.889
8	0.251	0.00	0.170
9	0.481	0.539	0.074
10	0.746	0.112	0.039
11	0.699	0.123	0.001
12	0.307	0.108	0.049
13	0.953	0.146	0.062
14	0.371	0.566	0.421
15	0.433	0.550	0.235

[a] Boxed values indicate a positive study result. The optical density (OD) values for a positive ELISA result were 0.241, 0.230, and 0.221 for feces from subjects infected with pathogenic zymodemes (PZ) and nonpathogenic zymodemes (NPZ) (PZ + NPZ) and feces from subjects infected with pathogenic zymodemes (PZ feces) and for serum from subjects with pathogenic and nonpathogenic zymodemes (PZ + NPZ), respectively.

feces from 8 of the 15 Egyptian subjects had pathogenic zymodeme-specific GIAP (Table 1). Seven of these eight subjects had GIAP antigen in their sera, whereas none of seven subjects with presumed nonpathogenic intestinal infection had GIAP antigen in their sera ($P < 0.05$ by the Fisher exact test).

DISCUSSION

We reported the successful detection of *E. histolytica* GIAP heavy-subunit antigen in serum by ELISA using epitope-specific monoclonal antibodies. By correlation with studies of stool culture, clinical status, and detection of GIAP antigen in feces, we found that detection of GIAP antigen in serum is a highly specific marker for infection by pathogenic *E. histolytica*. In fact, even asymptomatic infection with a pathogenic zymodeme results in serum antigenemia. We demonstrated by SDS-PAGE and immunoblotting that the intact GIAP heavy subunit is present in sera. Results of the present study suggest that a rapid and specific diagnosis of potentially invasive pathogenic infection can be accomplished by using ELISA technology on serum or fecal samples. The low incidence of false-positive serum antigen studies in U.S. controls and sera collected from controls residing in areas endemic for *E. histolytica* such as Egypt and South Africa and the low negative predictive values (0.01) indicate that detection of GIAP antigen in serum has potential for clinical use.

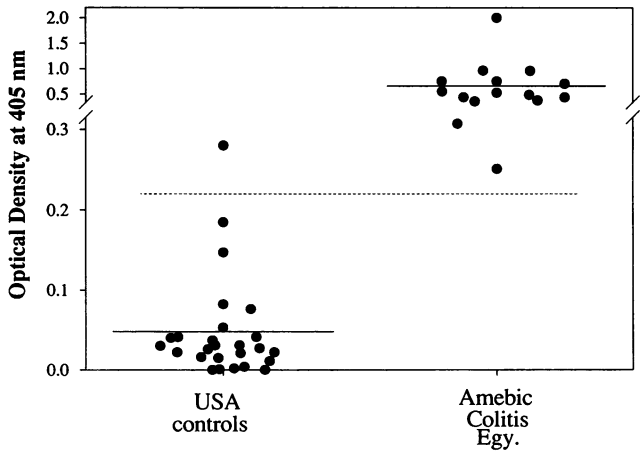

FIG. 4. Detection of GIAP antigen in feces by ELISA. The monoclonal antibodies used in the present study (3F4 and 8A3) detect both GIAP antigen from pathogenic and nonpathogenic isolates. Sera from only 1 of 26 U.S. controls were positive, whereas sera from all 15 Egyptians with amebic colitis were positive ($P < 0.01$).

Previously, we demonstrated that GIAP is a highly conserved parasite antigen. Native GIAP purified from a single clone of the HM1:IMSS strain, originally isolated in Mexico City, is recognized by antibodies in greater than 95.0% of sera from over 400 subjects with invasive amebiasis residing in diverse geographic areas such as Mexico, the United States, Canada, South Africa, India, and Egypt (1, 18, 21). These studies form the basis for the hypothesis that detection of antigen in feces and serum with anti-GIAP monoclonal antibodies could be useful in the diagnosis of amebiasis. Monoclonal antibodies specific for the *E. histolytica* GIAP were useful in characterizing and purifying this molecule (16, 19, 22). Using these antibodies, Petri et al. (17) identified distinct epitopes present in GIAP antigen from nonpathogenic and pathogenic isolates, making possible the specific ELISAs used in the present study.

Previously, amebic antigen in serum was detected exclusively in immune complexes by using polyclonal antibody to total parasitic proteins (5, 20). These methods required precipitation of immune complexes by PEG by using relatively large volumes of sera and were not specific for defined *E. histolytica* antigens. To our knowledge, this is the first report of the direct detection of a specific *E. histolytica* antigen in sera. The ELISA used to detect antigen in serum

used monoclonal antibodies which also recognize the GIAP present in nonpathogenic isolates (17). However, studies of sera from Egyptian and South African subjects demonstrated that the presence of GIAP in sera is specific for infection with pathogenic organisms. This correlates with our previous findings that nonpathogenic *E. histolytica* infection does not elicit an antibody response in serum (12, 21), suggesting a lack of systemic antigenic exposure. Only a single serum sample was collected from each subject with intestinal infection; therefore, we cannot correlate the presence of GIAP in serum with the duration of infection or the clinical course of infection.

Numerous groups have been successful in using ELISA with polyclonal antiamebic antibodies or uncharacterized monoclonal antibodies to detect *E. histolytica* antigen in feces (9, 10, 15, 29). Those reports did not differentiate pathogenic from nonpathogenic infections and therefore were of limited clinical utility. Use of a monoclonal antibody to 84- and 81-kDa proteins in immunofluorescence assays of isolates cultured from stool specimens was successful in identifying individuals with pathogenic infections (8). Culture and immunofluorescence are cumbersome, time-consuming procedures requiring specialized laboratory support. While our studies were in progress, Haque et al. (11) reported the detection of GIAP antigen in feces using an ELISA with rabbit polyclonal anti-GIAP "catching antibodies" and 7F4 and 8C12 monoclonal antibodies as secondary antibodies, which were identical to the monoclonal antibodies used in the present study. This system was specific for pathogenic isolates and was successful in detecting GIAP in fecal samples from 12 subjects with pathogenic infections. We have extended these findings by using an entirely monoclonal antibody-based system that was successful in detecting GIAP antigens in fecal samples from subjects infected with both pathogenic and nonpathogenic organisms. By correlating studies of fecal antigen detection with serum antigen detection, we confirmed the fact that the ELISA distinguishes between pathogenic and nonpathogenic infections. The methodology reported here may be more useful for epidemiologic studies of intestinal amebiasis, while it has clinical utility equal to those of the methods described by Haque et al. (11).

The Egyptian subjects who we studied were consecutive individuals who presented for study at a tropical disease clinic. Despite this biased population, we were surprised that such a high percentage of Egyptians with asymptomatic amebic infection (42%) had serum antigenemia, indicating a pathogenic-type *E. histolytica* infection. This is consistent with a recent report by Acuna-Soto et al. (2) of *E. histolytica* infection in a rural community in Mexico. Those authors identified pathogenic infection by amplification of fecal DNA samples using polymerase chain reaction and hybridization with polymerase chain reaction products. In that prospective study, all but 1 subject harbored pathogenic *E. histolytica* isolates, and 14 subjects (56%) had mixed infections. The results of that study and ours are in contrast to those of another study that found that only 10% of *E. histolytica*-infected subjects in South Africa harbored pathogenic isolates. There are wide regional variations regarding the percentage of subjects infected with potentially invasive pathogenic isolates. Results of the studies mentioned above further emphasize the need for diagnostic techniques to distinguish pathogenic from nonpathogenic *E. histolytica* infections. In areas endemic for *E. histolytica* it is not a common practice to treat individuals with asymptomatic *E. histolytica* infection. However, this strategy may not be appropriate in specific regions where pathogenic organisms predominate. There is general agreement that intestinal infection with a known pathogenic isolate warrants treatment (7, 12).

We have previously demonstrated that a significant percentage of uninfected individuals in areas endemic for *E. histolytica* have serum antibodies to GIAP, apparently because of prior infection with pathogenic *E. histolytica*. Serum antiamebic immunoglobulin G antibodies are long-lived, and therefore, their use in the diagnosis of active pathogenic *E. histolytica* infection is limited. Recently, Zhang et al. (30) studied serum antibody responses to a nonglycosylated recombinant form of the GIAP heavy subunit produced by a polymerase chain reaction based on the nucleotide sequence reported by Tannich et al. (27). They found that controls in an area endemic for *E. histolytica* had a low incidence (2 of 93) of serum anti-recombinant GIAP heavy-subunit antibodies. This is a promising finding that might improve the clinical utility of serology studies. Nevertheless, detection of antigen in both sera and feces provides a potentially optimal system for the diagnosis of both intestinal and hepatic amebiases. A prospective study is needed to determine the duration of antigenemia in serum following treatment of intestinal amebiasis and amebic liver abscess. Our current findings indicate that antigenemia in serum is not persistent because of the low positivity rate in uninfected controls from the same region. The ELISA technology used in the present study does not require extensive laboratory facilities, and studies with defined monoclonal antibodies can be reproduced worldwide.

In summary, detection of a highly conserved *E. histolytica* antigen, the GIAP heavy subunit, in feces and sera holds promise for increasing our ability to diagnose asymptomatic pathogenic infections and invasive amebiasis. The differentiation between pathogenic and nonpathogenic intestinal infections should enhance the effectiveness of physicians in the management of amebiasis, especially in areas highly endemic for *E. histolytica*.

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