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Serologic Reactivity to Purified Recombinant and Native 29-Kilodalton Peripheral Membrane Protein of Pathogenic *Entamoeba histolytica*

BECKY M. FLORES,[1] SHARON L. REED,[2] JONATHAN I. RAVDIN,[3] AND BRUCE E. TORIAN[1]*

Department of Pharmaceutical Sciences, College of Pharmacy, Idaho State University, Pocatello, Idaho 83209[1]; Division of Infectious Diseases, University of California, San Diego, San Diego, California 92103[2]; and Department of Medicine, Case Western Reserve University, and Cleveland Veterans Affairs Medical Center, Cleveland, Ohio 44106[3]

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The 29-kDa peripheral membrane protein of *Entamoeba histolytica* has recently been demonstrated to have epitopes on pathogenic clinical isolates which were not detected by monoclonal antibodies on nonpathogenic isolates. To analyze the serological response to this protein, we tested 93 serum specimens (from 33 patients with amebic liver abscess, 7 patients with colitis, 2 patients with ameboma, 18 individuals harboring a nonpathogenic zymodeme strain, 10 healthy Mexican migrant workers, and 23 healthy controls) by enzyme-linked immunosorbent assay (ELISA) using immunoaffinity-purified native or recombinant protein. When tested by ELISA with the native antigen, 79% (26 of 33) of the serum specimens from patients with amebic liver abscess, 4 of 9 serum specimens from symptomatic patients with colitis or ameboma, and serum from one migrant worker were positive. None of the 18 subjects harboring a nonpathogenic strain or 23 control individuals were seropositive to the native antigen (sensitivity, 71%; specificity, 98%). Of 30 serum specimens from patients with amebic liver abscess tested with recombinant antigen, 27 were seropositive (90%). In addition, six patients with colitis or ameboma and two individuals who harbored a nonpathogenic strain were seropositive to the recombinant antigen. One healthy Mexican migrant worker tested positive by both ELISAs (sensitivity, 87%; specificity, 94%). Immunoblotting of 51 serum specimens to sodium dodecyl sulfate-denatured native 29-kDa protein was less sensitive (65%) than ELISA in detecting serum antibodies to the antigen. These results suggest a similar antibody response to native and recombinant antigens ($r = 0.86$) and support the potential utility of a quantitative assay with defined recombinant antigen for the serodiagnosis of invasive amebiasis in nonendemic areas in conjunction with other diagnostic tools.

Entamoeba histolytica is a major cause of morbidity and mortality worldwide (22). Diagnosis of amebic infection is made by the identification of trophozoites or cysts in stool, a laborious and insensitive test. In addition, trophozoites of nonpathogenic zymodemes with low potential for invasion (14) are morphologically identical to *E. histolytica* with pathogenic zymodemes. Serological assays, including indirect hemagglutination, agar gel diffusion (AGD), counterimmunoelectrophoresis (CIE), or enzyme-linked immunosorbent assay (ELISA) with extracts of trophozoites, have proven useful but may be negative early in the course of invasive disease or remain positive for years (5), thus limiting the sensitivities of the tests for the diagnosis of acute invasive disease. Diagnostic tests with defined purified antigens (7, 9, 11, 16, 18, 20, 23), DNA probes specific for pathogenic or nonpathogenic zymodemes (4), or antigen-capture assays with monoclonal antibodies (8) are potentially more sensitive and specific. The characterization of surface antigenic species which stimulate a specific antibody response in patients with amebiasis is critical for defining important components which might be useful in a combined recombinant antigen serological test. We recently characterized the cDNAs that encode a 29-kDa surface antigen of *E. histolytica* whose epitopes allowed differentiation of pathogenic and nonpathogenic *E. histolytica* with monoclonal antibodies (10, 19). In the present study, we performed a quantitative ELISA with purified native or recombinant antigen and immunoblotting in order to determine the serological response to the 29-kDa protein. To our knowledge, this is the first report comparing the serum reactivity to purified native and recombinant antigens of *E. histolytica*.

MATERIALS AND METHODS

Cultivation of organisms and purification of native antigen. *E. histolytica* ATCC 30887 was cultivated in Diamond's TYI-S-33 (2) supplemented with 10% heat-inactivated bovine serum (BioFluids, Rockville, Md.). Trophozoites were harvested in the mid-logarithmic phase of growth as reported previously (10). Antigen was purified by immunoaffinity chromatography with four protein A-purified monoclonal antibodies specific for the 29-kDa protein (FP 4, FP 10, FP 11, FP 21), as described previously (3). Immediately after purification, the fractions were dialyzed against 10 liters of 0.01 M phosphate-buffered saline (PBS; pH 7.6). Protein concentrations were estimated by using the bicinchoninic acid reagent (Pierce, Rockford, Ill.) and bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.) as standards. Antigen purity was assessed by silver staining.

Human sera. Blood samples were obtained from 93 individuals: 42 patients with invasive amebiasis (33 with amebic liver abscess, 7 with colitis, 2 with ameboma), 18 individuals harboring a nonpathogenic zymodeme strain, 10 healthy Mexican migrant workers working in Virginia who had no documented history of amebic disease, and 23 healthy U.S.

* Corresponding author.

individuals (controls). Serum specimens from 13 of the patients with an hepatic abscess and 8 of the asymptomatic subjects infected with a nonpathogenic strain were collected in South Africa. All other serum samples were collected from individuals residing in the United States. Amebic liver abscess was diagnosed by clinical syndrome, positive ultrasound or computerized tomography, positive serology by AGD, CIE, or ELISA to total parasite protein, positive liver aspirate and culture, and positive response to specific antiamebic therapy. Three of the healthy Mexican migrant workers were seropositive to the adherence protein (9). The 23 healthy controls had no previous history of amebiasis. Stool samples from 13 of the controls were examined for ova and parasites and were negative for *E. histolytica* cysts or trophozoites. Two of the stool samples contained *Entamoeba coli* cysts and trophozoites, and one had *Giardia lamblia* cysts. The controls were either seronegative by indirect hemagglutination (titer, ≤2) or seronegative by ELISA to the *E. histolytica* native adherence protein (9). All serum samples were heat inactivated at 56°C for 30 min before use.

Expression and purification of recombinant protein. The full-length cDNA insert which encodes the 29-kDa protein (10) was subcloned in the pGEX expression vectors (17). The presence of a ribosomal binding site in the cDNA (10) allowed the expression of the protein in all three vectors, not as a fusion protein but as a 29-kDa protein (1). The recombinant protein expressed in pGEX-3X was used for purification. Briefly, a culture of transformed *Escherichia coli* DH5α was treated essentially as described previously (19), except that the cells were disrupted by using a French press at 20,000 lb/in^2 (SLM Instruments, Inc., Urbana, Ill.), and the recombinant protein was purified by immunoaffinity chromatography by using a column prepared exclusively for the purification of recombinant protein. After initial purification, the sample was dialyzed in PBS and then applied to a glutathione agarose (Sigma) column which selectively removed any fusion protein which may have been synthesized. The unbound fractions containing the recombinant 29-kDa protein were concentrated and used for the assays.

ELISA. For ELISA, 96-well flat-bottom microtiter plates (Immulon II; Dynatech, Alexandria, Va.) were incubated with 100 ng of immunoaffinity-purified or recombinant protein per well (2 μg/ml) at 4°C overnight. Unbound antigen was removed, and the wells were blocked with 125 μl of PBS containing 7.5% bovine serum albumin (BSA) at 37°C for 1 h. The blocking reagent was removed and the wells were incubated with 50 μl of patient serum diluted 1:500 in blocking buffer for 1 h at 37°C. The plates were washed four times in PBS containing 0.05% Tween 20 (PBST), and then a 1:1,500 dilution of horseradish peroxidase-conjugated goat anti-human immunoglobulin A (IgA), IgM, and IgG antibodies (Zymed, South San Francisco, Calif.) was added to each well (50 μl per well in blocking buffer). The plates were incubated for 1 h at 37°C, washed four times in PBST and two times in PBS, and then developed with *o*-phenylaminediamine and hydrogen peroxide. The reactions were allowed to develop for 10 min in the dark and were then terminated by the addition of 25 μl of 8 N sulfuric acid per well. The A_{492} was measured on a Titertech Multiskan Mk II ELISA plate reader (ICN/Flow, McLean, Va.). Assays were performed in triplicate wells, and the means were determined after subtracting the measurement from test wells to which no antibody was added.

Immunoblotting. Immunoaffinity-purified native protein (0.7 μg) was electrophoresed on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels (6) and electroblotted onto nitrocellulose membranes (21). The nitrocellulose membrane was cut into strips and blocked with 7.5% BSA in 20 mM Tris-Cl (TB; pH 7.5) and 0.15 M NaCl at 4°C overnight. Sera were diluted 1:500 in TB containing 5% milk and 0.1% Tween 20 and were incubated with the strips for 1 h at room temperature. After six washes in TB containing 0.5 M NaCl and 0.2% Tween 20, the strips were incubated with horseradish peroxidase-conjugated anti-human IgA, IgG, and IgM (1:3,000 in antibody diluent; Zymed) for 1 h. After washing, the strips were incubated with the enhanced chemiluminescence reagent (Amersham, Arlington Heights, Ill.) according to the manufacturer's recommendations. The strips were exposed to X-ray film (Kodak X-OMAT SB-5; Sigma) for 10 s and were then developed. Reactivity to the 29-kDa band was scored as strong, moderate, weak, or negative. After the initial scoring, negative blots were exposed to film for up to 1 min, with no change in the result. In addition to using specific monoclonal antibody as a control, representative known positive and negative sera were also included in each experiment.

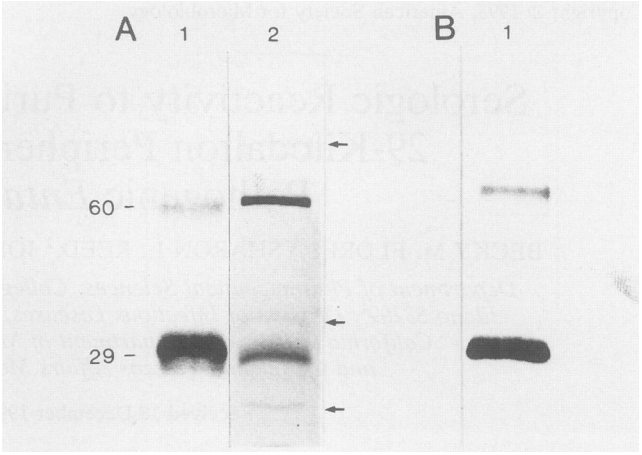

FIG. 1. Assessment of antigen purity. (A) Immunoaffinity-purified native (lane 1) and recombinant (lane 2) proteins were electrophoresed on a 10% SDS-polyacrylamide gel and were then silver stained. Both preparations showed a 29-kDa and a 60-kDa band. Three faintly stained bands were also detected in the recombinant protein preparation (indicated by arrows). (B) Immunoblot of the recombinant protein (1 μg) with monoclonal antibody specific for the 29-kDa protein demonstrating immunoreactive bands.

RESULTS

Purity of antigen preparation. To assess the purity of the immunoaffinity-purified native or recombinant protein used in ELISA and immunoblotting assays, protein samples were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions; this was followed by silver staining. Native, affinity-purified antigen showed two bands: a prominent 29-kDa band and a minor 60-kDa band (Fig. 1A, lane 1). The 60-kDa band was previously shown to be the dimeric form of the 29-kDa protein and was immunoreactive with monoclonal antibody (3). Analysis of the purified recombinant protein on a silver-stained gel also revealed the 29- and 60-kDa bands and three very faintly stained bands (Fig. 1A, lane 2). Immunoblotting of the recombinant protein with specific monoclonal antibody demonstrated that the faint bands were not immunoreactive (Fig. 1B). Although the procedure used for purification of the recombinant protein

involved the use of two chromatography columns, the copurification of minor amounts of *E. coli* components cannot be ruled out.

Serological reactivity to the native and recombinant 29-kDa proteins by ELISA. To assess the human antibody response to the 29-kDa protein, we tested individual serum samples by an ELISA using immunoaffinity-purified native (n = 93) or recombinant (n = 89) protein as the antigen. The mean of the absorbance measurements of sera from the 23 healthy controls plus three standard deviations was used as the cutoff for a positive test. Measurements greater than 0.357 or 0.109 were considered positive for native or recombinant antigen assays, respectively. From patients who had amebic liver abscess disease, 26 of 33 (79%) serum samples reacted with the native protein, while 27 of 30 (90%) serum samples reacted with the recombinant protein (Fig. 2A and B). Because of insufficient quantities, serum specimens from three patients from South Africa with amebic liver abscess which were positive to the native antigen and from one Mexican migrant worker which was negative to the native antigen were not tested with the recombinant antigen. The three serum specimens from patients with amebic liver abscess which did not react with native or recombinant protein were negative by the AGD test. Among the serum specimens from the nine patients with colitis or ameboma, four (44%) reacted with the native protein and seven (78%) reacted with the recombinant protein. All 18 serum specimens from patients infected with a nonpathogenic strain were seronegative when tested with the native antigen, but two of the eight serum specimens obtained from South African patients tested positive with the recombinant antigen. One sample obtained from a healthy Mexican migrant worker which was seropositive to the adherence protein was seropositive by both 29-kDa antigen assays. Sera from all 23 healthy controls showed no reactivity to native or recombinant protein.

In the present study, sera from most patients infected with pathogenic *E. histolytica* strains (71%) were seropositive to the native antigen (sensitivity, 71%), while only 1 of 51 (1.9%) serum specimens from healthy controls, healthy Mexican migrant workers, or individuals harboring a nonpathogenic strain was positive (specificity, 98%). The use of recombinant protein in the assay resulted in a greater sensitivity in detection (87%) but a decrease in the specificity (94%). Overall, measurements from the assay with recombinant antigen correlated with measurements from the assay with native antigen (Fig. 3; correlation coefficient, r = 0.86). Both assays showed differences in the reactivities of sera from patients infected with pathogenic strains (symptomatic group) and the asymptomatic group (healthy controls, Mexican migrant workers, and those who harbored a nonpathogenic strain) $P < 0.001$, Student's t test). Analysis of variance of the group means of uninfected individuals and those with a nonpathogenic strain showed no difference (P = 0.5).

Reactivities of sera to the native protein detected by immunoblotting. To examine the potential differences between the reactivities of serum antibodies to nondenatured protein (ELISA) and denatured protein, we tested sera with SDS-denatured native 29-kDa protein. Because insufficient amounts of sera were available from the South Africans, the migrant worker group, and 11 of the controls, we were able to test only 51 serum samples. The sera were tested at a single dilution (1:500), and reactivity was visualized on X-ray film. Thirteen of 20 (65%) serum specimens from patients with amebic liver abscess showed reactivity to the 29-kDa band (Fig. 4). Of the nine serum specimens from patients with colitis or ameboma, only one (colitis) was positive by immunoblotting. Sera from 12 healthy U.S. controls and 10 individuals infected with a nonpathogenic strain did not react with the 29-kDa protein.

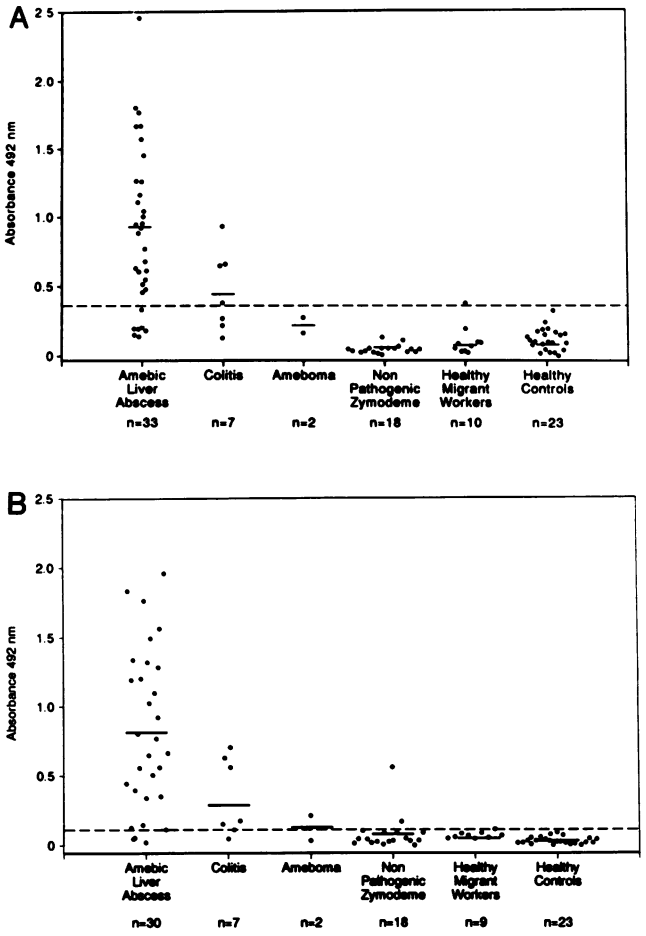

FIG. 2. Detection of antibodies to immunoaffinity-purified native and recombinant proteins by ELISA. Group means are indicated by a horizontal bar. The broken horizontal line represents three standard deviations above the mean of the healthy control group that was used as a cutoff for a positive result. (A) ELISA with immunoaffinity-purified native protein as the antigen. Mean A_{492} values were 0.899 ± 0.579 for patients with amebic liver abscess, 0.465 ± 0.291 for patients with colitis, 0.196 ± 0.113 for patients with ameboma, 0.052 ± 0.031 for patients infected with a nonpathogenic strain, 0.11 ± 0.107 for healthy migrant workers, and 0.12 ± 0.079 for healthy controls. The cutoff for a positive result was 0.357. (B) ELISA with purified recombinant protein as the antigen. Mean A_{492} values were 0.819 ± 0.578 for patients with amebic liver abscess, 0.34 ± 0.28 for patients with colitis, 0.124 ± 0.126 for patients with ameboma, 0.081 ± 0.128 for patients infected with a nonpathogenic strain, 0.072 ± 0.021 for healthy migrant workers, and 0.034 ± 0.025 for healthy controls. The cutoff for a positive result was 0.109.

DISCUSSION

Definition of the serologically relevant antigens of *E. histolytica*, especially those which possess pathogenic zymodeme-specific epitopes, is critical to the understanding of the humoral response to amebic infection, the development

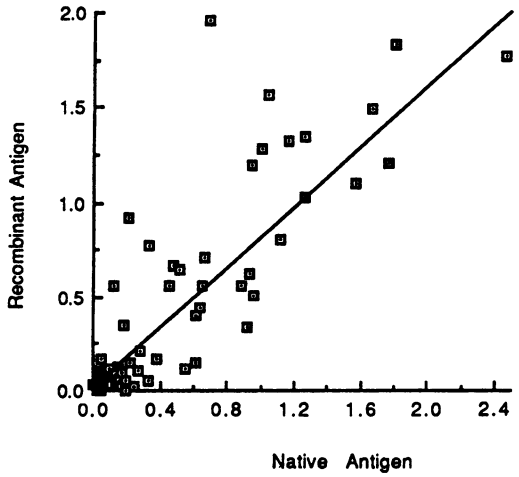

FIG. 3. ELISA measurements with recombinant antigen versus ELISA measurements with native antigen. The same serum samples (n = 89) were tested in both assays, and tests with both antigens showed a high degree of agreement (r = 0.86).

of new diagnostic tests, and the identification of potential vaccinogens. Although the antibody response does not appear to be involved in protection from invasive disease (13), detection of specific antibodies is very useful in diagnosing invasive amebic disease. A number of serologic studies with pure or mixed antigens of *E. histolytica* have been shown to be very sensitive for the detection of antibodies to *E. histolytica* in the serum of patients with invasive amebiasis (7, 9, 11, 12, 15, 16, 18, 20, 23). Some assays also detected antibodies in the sera of patients with a nonpathogenic strain

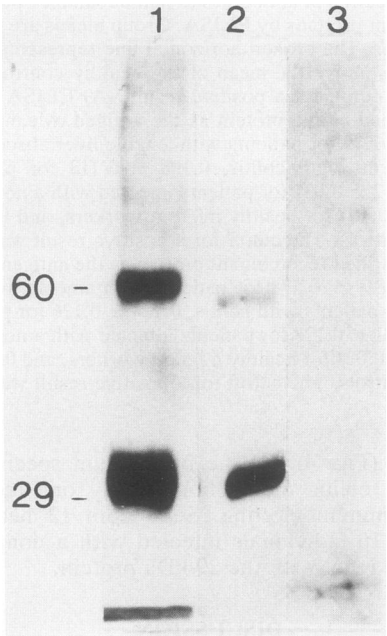

FIG. 4. Immunoblot showing the reactivities of representative sera to purified native 29-kDa protein (0.7 μg). Sera from patients with an amebic liver abscess showing a strong (lane 1) or moderate (lane 2) antibody reactivity to the antigen. Serum from a control individual showed no reactivity to the antigen (lane 3).

or in the sera of those who were culture negative for *E. histolytica* (9, 15); however, these studies were conducted on sera collected in an area endemic for *E. histolytica* and prior infection with pathogenic *E. histolytica* could not be ruled out.

Although one of the healthy Mexican migrant workers was seropositive, no antibody response to the native 29-kDa antigen was detected in 23 controls or patients who were from areas not endemic for *E. histolytica* and who harbored nonpathogenic strains. The absorbance measurements in assays of sera from patients with intestinal amebiasis suggested an apparent lower antibody response than those in assays of sera from patients with hepatic amebiasis. This observation may be a result of the small sample size tested or may be due to a poor serological response. The lack of reactivity of sera from two patients with ameboma might reflect a low antigen load because both patients had a positive CIE test result but a negative AGD test result.

Eight additional positive ELISA results were detected when recombinant protein was used as the antigen. Six of the serum specimens which reacted with recombinant antigen were from patients who had symptomatic infections. The other two positive serum specimens were from South Africans who harbored a nonpathogenic strain; however, it is not known whether they had a previous infection with pathogenic *E. histolytica*. The increased sensitivity and lower specificity of the ELISA with recombinant antigen may be due to the presence of minor *E. coli* components which may have been copurified with the recombinant *E. histolytica* protein. Differences in presentation by native and recombinant antigens may also affect antibody reactivities. Overall, the serologic response to the recombinant antigen from all samples tested was similar to the results obtained in assays with native antigen. Recently, a serological study performed by ELISA with a purified 29-kDa surface protein of *E. histolytica* as the antigen showed that serum specimens from 100% (n = 13) of patients with amebic liver abscess and all five serum specimens from asymptomatic cyst carriers were positive, while healthy subjects were seronegative (16). Since the nucleotide sequence for the protein purified by these investigators has not been reported, it is currently unknown whether the protein which they described is identical to the 29-kDa antigen that we used in the present study.

Although immunoblotting of the pure protein appeared to be a less sensitive test than ELISA, even when enhanced chemiluminescence was used for detection, serum antibodies may be more likely to recognize native antigen than SDS-denatured antigen. The lack of reactivity to the denatured antigen may reflect alteration of epitopes. Alternatively, this result may reflect the time during the course of infection when serum was collected. Generally, the sensitivities of immunoblotting assays have been lower when compared with those of standard assays for serodiagnosis (18, 20), but immunoblotting assays have also been shown to be more specific for sera obtained during the acute phase of illness (23).

The sensitivity and specificity obtained with recombinant antigens in ELISA and/or immunoblotting for the serodiagnosis of invasive amebiasis have recently been demonstrated for an immunodominant 125-kDa protein and the 170-kDa adherence protein of *E. histolytica* (7, 23). The present study showed that an ELISA with recombinant 29-kDa antigen is also very sensitive in detecting antibodies in symptomatic patients and is the first to compare a quantitative response to both the native and recombinant antigens of *E. histolytica* by ELISA, showing that serum antibody recognition of the

recombinant 29-kDa surface protein is similar to that observed with native antigen. Comparison of antibody responses to native and recombinant antigens is an important aid in the analysis of other studies involving immunological criteria when recombinant antigen is used to ensure that a host response is similar between recombinant and native antigens (B- or T-cell mediated). Serologic assays with several defined surface antigens will likely provide greater specificities than currently available assays, and the use of recombinant antigens will allow large-scale production of well-defined antigens. Epitope mapping with recombinant antigen is being performed to define those regions of the 29-kDa antigen which are immunogenic.

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