Use of a Recombinant 170-Kilodalton Surface Antigen of *Entamoeba histolytica* for Serodiagnosis of Amebiasis and Identification of Immunodominant Domains of the Native Molecule

YUYING ZHANG,^{1†} ELLEN LI,^{1,2} TERRY F. H. G. JACKSON,³ TONGHAI ZHANG,¹
VINODH GATHIRAM,³ AND SAMUEL L. STANLEY, JR.^{1,4*}

Departments of Medicine,¹ Biochemistry and Molecular Biophysics,² and Molecular Microbiology,⁴
Washington University School of Medicine, St. Louis, Missouri 63110, and Research Institute
for Diseases in a Tropical Environment, Congella, Durban 4013, Republic of South Africa³

Received 10 June 1992/Accepted 20 August 1992

We expressed the gene that encodes one of the major surface antigens of *Entamoeba histolytica*, the 170-kDa protein (1,270 amino acids), as a glutathione *S*-transferase fusion protein containing amino acids 1 to 1202 (lacking the putative transmembrane and cytoplasmic regions) and as separate fusion proteins containing each of three major domains of the 170-kDa molecule. Lysates from bacteria induced to express one of these proteins were used as the target antigens in a Western blot (immunoblot) analysis to determine whether a recombinant 170-kDa antigen could serve as the basis for a serologic test used to detect invasive amebiasis and whether there are differences in humoral immunogenicity among the three major domains of the 170-kDa antigen. Among patients with invasive amebiasis from three major areas where the disease is endemic and two sites in the United States, 54 (90%) of 60 had antibodies to the recombinant 170-kDa protein. Among 37 patients from regions where the disease is endemic and 20 patients from the United States without amebic disease, 1 (2%) of 57 had antibodies to the recombinant 170-kDa protein. We found significant differences in seroreactivity to each of three major domains of the molecule among patients seropositive for the complete construct, ranging from 100% seroreactivity with the fusion protein containing the domain designated cysteine rich and 89% seropositivity with the fusion protein incorporating a portion of the region designated cysteine poor to only 9% seropositivity for the fusion protein containing the pseudorepeat domain. Our study indicates that a serologic test based on the recombinant 170-kDa antigen could serve as a highly sensitive and specific test for acute invasive amebiasis.

The protozoan pathogen *Entamoeba histolytica* is a major cause of debilitating illness and death worldwide. Yearly, more than 40,000,000 people develop invasive amebiasis, which is manifested most commonly as diarrheal disease and less frequently as amebic liver abscess (13). Examination of amebic serology is a critical component in the diagnosis of invasive amebiasis. Conventional serologic tests such as the indirect hemagglutinin test are highly sensitive for detection of invasive amebic disease, but seropositivity can persist for years (4, 5). A number of studies have demonstrated that between 6 and 20% of healthy subjects in regions where invasive amebiasis is endemic have a positive serologic diagnosis, probably secondary to prior infection with pathogenic *E. histolytica* (2, 3, 5). This high background level of seropositivity in regions where the disease is endemic can complicate the serologic diagnosis of acute amebic disease.

Recently we described the use of a recombinant *E. histolytica* protein as the target antigen in a Western blot (immunoblot) test used to diagnose invasive amebiasis (9, 10). In this retrospective study, Western blotting using the 52-kDa recombinant serine-rich *E. histolytica* protein proved to be a highly specific test for invasive disease and had a higher positive predictive value (92 versus 65%) than a conventional serologic test, agar gel diffusion, for diagnosis

of acute amebiasis. However, the overall sensitivity of the serine-rich *E. histolytica* protein-based test (82%) was lower than the 90 to 100% sensitivity reported for conventional tests (10). To improve upon a recombinant-antigen-based test, we expressed one of the major surface antigens of *E. histolytica*, the 170-kDa surface molecule (6, 11), as a pGEX-encoded fusion protein in *Escherichia coli*. A fusion protein containing 1,202 of the native protein's 1,270 amino acids and fusion proteins containing three major domains of the molecule were used as the target antigens in a Western blot test. We describe herein our findings that Western blot analysis using a recombinant 170-kDa antigen may prove to be a highly sensitive and specific test for acute invasive amebiasis and that significant differences in seroreactivity to the three major domains of the recombinant molecule exist among patients with amebiasis.

MATERIALS AND METHODS

PCR. The polymerase chain reaction (PCR) was performed under standard conditions on genomic DNA from *E. histolytica* HM1:IMSS by using primers derived from the nucleotide sequence of a gene that encodes the 170-kDa antigen described by Tannich et al. (11). The primers used to obtain the 3,606 nucleotides that encode amino acids 1 through 1202 of the 170-kDa protein were 5'-gtaggcctgataaa cctaatagaatttca3' and 5'-attataatttctcctgcgag3'.

Construction of pGEX-KG hybrid gene fusions. For construction of fusion proteins, the pGEX-KG vector was used;

* Corresponding author.

† Present address: Institute of Microbiology, Academica Sinica, Beijing, 100080 People's Republic of China.

FIG. 1. The 170-kDa antigen-encoding region of the pGEX-170 construct. The *Bgl*II sites are marked, as are the regions that encode the cysteine-poor, pseudorepeat, and cysteine-rich regions of the 170-kDa protein as described by Tannich et al. (11). The relative positions of the nucleotides cloned into each of the pGEX constructs (pGEX-170CP, pGEX-170PR, and pGEX-170CR) are indicated.

this encodes a fusion protein in which the 170-kDa antigen is preceded by glutathione *S*-transferase protein (27 kDa) (1). The PCR product was ligated into the PCR1000 TA cloning vector product and designated PCR170. The insert was isolated by digesting PCR170 with *Stu*I and *Hind*III and ligated with a blunt-*Hind*III large fragment of pGEX-KG. The pGEX-KG fragment was prepared by digestion with *Eco*RI, filling in of the resulting 3' recessed termini with the Klenow fragment of DNA polymerase I, and then digestion with *Hind*III. This construct was designated pGEX-170. A schematic of the 170-kDa antigen-encoding portion of the pGEX-170 construct indicating the *Bgl*II sites; the locations of nucleotides that correspond to the cysteine-poor, cysteine-rich, and pseudorepeat regions of the native protein (11); and the nucleotides cloned into each of the subsequent constructs is shown in Fig. 1. pGEX-170CP, which encodes amino acids 1 to 373 and contains the region of the 170-kDa protein designated cysteine poor by Tannich et al. (11), was constructed by digesting pGEX-170 with *Bgl*II and *Hind*III and filling in the resulting 3' ends with Klenow. The large fragment was gel purified and self-ligated. pGEX-170PR, which encodes amino acids 374 to 648 (within the region of the 170-kDa protein designated pseudorepeats [11]), was constructed by digesting pGEX-170 with *Bgl*II, filling in the resulting 3' end with Klenow, and ligating the appropriate fragment into pGEX-KG, which had been digested with *Eco*RI with the resulting 3' recessed termini filled in with Klenow. pGEX-170CR, which encodes amino acids 649 to 1202 and contains most of the region of the 170-kDa protein designated cysteine rich (11), was constructed by digesting pGEX-170 with *Bgl*II, filling in the resulting 3' end with Klenow, and digestion with *Hind*III. The resulting blunt-*Hind*III fragment was ligated into the blunt-*Hind*III large fragment of pGEX-KG described above. Expression of the fusion proteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of bacterial lysates induced with isopropyl- β -D-thiogalactopyranoside (1). In addition, Western blotting of SDS-PAGE-separated lysates with a polyclonal rabbit anti-*E. histolytica* serum (known to react with the native 170-kDa protein) and antiserum from a rabbit immunized with the glutathione *S*-transferase protein was used to identify fusion proteins (9).

DNA sequencing. Sequence analysis of PCR170 was performed by the dideoxy method by using primers derived from the published sequence of Tannich et al. (11). The pGEX constructs were sequenced by using primers derived

from the nucleotide sequence of the PCR170 fragment and the pGEX-KG vector (1).

Western blotting. Western blot analysis of insoluble bacterial lysates containing the fusion proteins was performed as previously described (10). All human serum samples were tested at a 1:500 dilution (10). All autoradiographs were exposed for 16 h. Blots were considered positive if binding to any of the 170-kDa fusion proteins was detected.

Patient population. The criteria for diagnosis of amebic liver abscess and details on the patient population and serum samples from Mexico; South Africa; San Diego, Calif.; and St. Louis, Mo., have been described previously (10). Serum samples were obtained from patients in Durban, Republic of South Africa (32 samples from 32 patients, 16 with amebic liver abscess and 16 with no evidence of amebiasis who were hospitalized with other illnesses); Mexico City, Mexico (26 serum samples from 26 patients with amebic liver abscess); San Diego, Calif. (7 serum samples from 2 patients with intestinal disease and 5 patients with amebic liver abscess); and St. Louis, Mo. (3 patients with amebic liver abscess, 20 patients without amebiasis). Twenty-nine serum samples obtained from patients seen in Hebei Province, People's Republic of China (7 serum samples from seven patients with amebic liver abscess, 1 sample from a patient with intestinal disease, 10 samples from controls from an area where the disease is endemic with no history of amebic disease, and 11 serum samples from asymptomatic *E. histolytica* cyst passers) were also analyzed. Detailed characteristics of this patient population have been previously reported (12).

Nucleotide sequence accession number. The nucleotide sequence of the PCR170 product has been given GenBank accession number L00636.

RESULTS

Sequence analysis of PCR products and constructs. Nucleotide sequence analysis of the 3.6-kb PCR product showed that it was 85% identical to the nucleotide sequence of the gene that encodes the 170-kDa antigen published by Tannich et al. (11) (data not shown). Most of the differences were in the most 5' 875 nucleotides. The nucleotide sequence was the same in three independently generated PCR products, suggesting that the differences from the published sequence result from polymorphisms in the gene(s) that encodes the 170-kDa protein rather than PCR error. The derived amino acid sequence was 93% identical to the derived amino acid sequence reported by Tannich et al. (11). Nucleotide sequence analysis of each of the pGEX-170 constructs revealed no nucleotide differences from the parent PCR fragment and demonstrated that each fragment was in frame with the pGEX fusion partner (data not shown).

Expression of pGEX-170 constructs. The results of SDS-PAGE-Coomassie analysis of cell lysates from isopropyl- β -D-thiogalactopyranoside-induced bacteria that express one of the four pGEX-170 constructs or the vector alone are shown in Fig. 2. The 27-kDa glutathione *S*-transferase product of the parental vector was in lane 1. A fusion protein of approximately 160 kDa encoded by the pGEX-170 construct was in lane 2, along with species at approximately 140, 98, 72, and 68 kDa which are presumed to represent truncated versions of the fusion protein or degradation products. An 80-kDa fusion protein encoded by the pGEX-170CP construct was in lane 3, along with a second band at 72 kDa. The 52-kDa product of the pGEX-170PR construct was in lane 4, along with a second band at 48 kDa, and the 110-kDa product of the pGEX-170CR construct, along with additional species

FIG. 2. Expression of the pGEX-170 constructs in *E. coli*. Lysates were prepared from identical numbers of isopropyl- β -D-thiogalactopyranoside-induced bacteria that express pGEX-KG (lane 1), pGEX-170 (lane 2), pGEX-170CP (lane 3), pGEX-170PR (lane 4), and pGEX-170CR (lane 5), run on SDS-PAGE, then stained with Coomassie blue. The locations of the fusion proteins are shown by arrowheads. Molecular size standards (kilodaltons) are indicated.

at 97, 72, and 68 kDa, was in lane 5. The identity of each of these species as a fusion protein was confirmed by Western blotting using polyclonal rabbit antiserum to the glutathione *S*-transferase protein and polyclonal rabbit anti-amebic serum (data not shown).

Seroreactivity with recombinant 170-kDa proteins. A total of 117 serum samples were examined for reactivity with lysates from cells that express parental vector pGEX, the construct pGEX-170, and the three constructs containing each of the major domains (pGEX-170CP, pGEX-170PR, and pGEX-170CR). Most of the positive blots showed sera which reacted with multiple bands in lanes containing fusion proteins encoded by pGEX-170, pGEX-170CP, and pGEX-170CR (Fig. 3A, lanes 2, 3, and 5) and did not react with the pGEX product (lane 1) or the pGEX-170PR-encoded fusion protein (lane 4). The most consistently reactive band was the 110-kDa product of the pGEX-170CR construct. Among all of the sera screened, only five samples were also reactive with the 52- and 48-kDa pGEX-PR-encoded fusion protein (Fig. 3B, lane 4). Most of the negative blots (Fig. 3C) showed no reactivity with any of the constructs; however, negative blots were seen in which serum samples reacted with species seen in all of the lanes (control vector as well as pGEX-170-containing constructs) (Fig. 3D) but did not react with any of the fusion proteins.

The results for all of the serum samples are summarized in Table 1. Among 60 patients with acute invasive amebiasis (57 with amebic liver abscess and 3 with intestinal disease), 54 (90%) had antibodies to the recombinant 170-kDa protein.

FIG. 3. Western blots of lysates from bacteria that express pGEX-170 constructs. (A) Serum from a patient with an amebic liver abscess. (B) Serum from a patient with an amebic liver abscess. (C) Serum from a disease-free patient in a region where amebiasis is endemic. (D) Serum from a disease-free patient in a region where amebiasis is endemic. For each panel, lane 1 contained lysates from bacteria that express pGEX, lane 2 contained lysates from bacteria that express pGEX-170, lane 3 contained lysates from bacteria that express pGEX-170CP, lane 4 contained lysates from bacteria that express pGEX-170PR, and lane 5 contained lysates from bacteria that express pGEX-170CR. Molecular size standards (kilodaltons) are indicated.

All 55 patients with antibodies to the recombinant 170-kDa protein had antibodies to the pGEX-170CR fusion protein, 49 of 55 had antibodies to the pGEX-170CP construct ($P < 0.025$ compared with seropositivity to pGEX-170CR, $\chi^2 = 6.4$ [1]), and 5 of 55 had antibodies to the pGEX-170PR construct ($P < 0.001$, $\chi^2 = 91.6$ [1], when compared with seropositivity to pGEX-170CR and $P < 0.001$, $\chi^2 = 70.4$ [1], when compared with seropositivity to pGEX-170CP). Among the 57 patients who did not have acute invasive amebiasis, 56 (98%) had no detectable antibodies to any of the recombinant 170-kDa proteins. Only one serum sample from a patient who did not have acute amebiasis showed reactivity with the recombinant 170-kDa protein.

TABLE 1. Seropositivity for the recombinant 170-kDa antigen

Diagnosis	No. of serum samples containing anti-pGEX-170 antibodies	% Positive
Amebic liver abscess	52/57	91
Amebic colitis	2/3	67
Invasive amebiasis (total)	54/60	90 ^a
Nonamebic disease	1/37	3
No disease (controls) ^b	0/20	0
No amebiasis (total)	1/57	2

^a $P < 0.001$ for the association of anti-pGEX-170 antibodies with invasive amebiasis ($\chi^2 [1] = 91.4$).

^b From an area where amebiasis is not endemic.

DISCUSSION

Two findings emerged from this study. The first was the discovery that a Western blot test using the recombinant 170-kDa antigen fusion protein could represent a highly sensitive and specific test for invasive amebiasis. The native 170-kDa protein has been extensively studied and is known to be immunogenic (reviewed in reference 7). A previous study using a monoclonal antibody and the purified native 170-kDa protein as the target antigen in immunoblotting and an enzyme-linked immunosorbent assay demonstrated high sensitivity for the test (99% seropositivity among patients with amebic liver abscess), but in the enzyme-linked immunosorbent assay, 25% of asymptomatic patients in regions where the disease is endemic (either asymptomatic cyst passers or uninfected patients) were seropositive as well (8). The Western blot test using the recombinant 170-kDa antigen showed somewhat less sensitivity than that seen in the native antigen tests (90%) but showed greatly improved specificity for the diagnosis of acute infection. Among 36 patients without acute disease (including 11 asymptomatic cyst passers and 7 patients with other illnesses who had positive conventional serologic tests) from two areas where amebiasis is endemic, only 1 was seropositive for the recombinant 170-kDa antigen. In this selected population, the positive predictive value for the recombinant 170-kDa antigen-based Western blot was 98%. One possible explanation for the seemingly different results seen with the recombinant protein compared with the monoclonal antibody-purified native 170-kDa antigen may lie in the lack of glycosylation of the recombinant protein. This suggests that antibodies to the 170-kDa protein that persist long after amebic infection recognize carbohydrate determinants on the native molecule.

We had previously shown that a recombinant serine-rich *E. histolytica* protein-based test possessed increased specificity for the diagnosis of acute invasive amebiasis compared with agar gel diffusion in a selected population of patients from South Africa but was less sensitive than the conventional test (10). The 170-kDa antigen-based test shows similar specificity (98%) but improved sensitivity (90%) compared with the serine-rich *E. histolytica* protein-based test (82%).

We also examined sera for reactivity with constructs containing three major domains of the 170-kDa lectin. Our data show that a portion of what has been designated the cysteine-rich region (amino acids 649 to 1202) appears to be immunogenic. Antibodies to this region were detected in every patient with invasive disease seropositive for the whole recombinant protein; a test based on this region alone

would be equivalent to a test based on the complete recombinant molecule. The region designated cysteine poor (amino acids 1 to 373) also appeared to be immunogenic, although seroreactivity with the recombinant protein containing this region was less than that seen with the pGEX-170CR-encoded protein. In contrast, only 9% of patients with invasive amebiasis who had antibodies to the pGEX-170 fusion protein had detectable antibodies to the pGEX-170-PR-encoded recombinant protein containing the region of the native molecule with pseudorepeats (amino acids 374 to 648). The reason for the apparent lack of immunogenicity of this region is unclear. The pseudorepeat region may be inaccessible in the natural conformation of the native molecule and thus nonimmunogenic; alternatively, this region of the native protein may possess conformationally dependent epitopes that result in antibodies which do not react with the denatured recombinant pseudorepeat fusion protein. We also cannot exclude the possibility that the primary sequence of this region is less conserved among different *E. histolytica* strains than are other portions of the native 170-kDa molecule. The difference in seroreactivity seen with three major domains of the recombinant molecule has implications in the design of a 170-kDa recombinant antigen-based test and may have implications for vaccine development using the recombinant 170-kDa protein.

ACKNOWLEDGMENTS

We thank Jesus Calderon and Sharon Reed for providing serum samples utilized in this study, Sharon Reed for helpful discussions of the manuscript, Thomas Steinberg for help with computer-generated figures, and Paul Cieslak for statistical analysis.

This work was supported in part by U.S. Public Health Service grant AI30084-01 and WHO MIM/15/181/174 to S.L.S. E.L. is the recipient of Research Career Development Award K04 DK02072. T.F.H.G.J. is supported by the South African Medical Research Council.

REFERENCES

1. Guan, K., and J. E. Dixon. 1991. Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* **192**:262-267.
2. Jackson, T. F. H. G., V. Gathiram, and A. E. Simjee. 1984. Serological differentiation between past and present infections in hepatic amoebiasis. *Trans. R. Soc. Trop. Med. Hyg.* **78**:342-345.
3. Jackson, T. F. H. G., V. Gathiram, and A. E. Simjee. 1985. Seroepidemiological study of antibody responses to the zymodemes of *Entamoeba histolytica*. *Lancet* **i**:716-719.
4. Krupp, I. M. 1970. Antibody response in intestinal and extra-intestinal amebiasis. *Am. J. Trop. Med. Hyg.* **19**:57-62.
5. Lobel, H. O., and I. G. Kagan. 1978. Seroepidemiology of parasitic diseases. *Annu. Rev. Microbiol.* **32**:329-347.
6. Mann, B. J., B. E. Torian, T. S. Vedvick, and W. A. Petri, Jr. 1991. Sequence of a cysteine-rich galactose-specific lectin of *Entamoeba histolytica*. *Proc. Natl. Acad. Sci. USA* **88**:3248-3252.
7. Ravdin, J. I. 1989. *Entamoeba histolytica*: from adherence to enteropathy. *J. Infect. Dis.* **159**:420-429.
8. Ravdin, J. I., T. F. H. G. Jackson, W. A. Petri, Jr., C. F. Murphy, B. L. Ungar, V. Gathiram, J. Skilogiannis, and A. E. Simjee. 1990. Association of serum antibodies to adherence lectin with invasive amebiasis and asymptomatic infection with pathogenic *Entamoeba histolytica*. *J. Infect. Dis.* **162**:768-772.
9. Stanley, S. L., Jr., A. Becker, C. Kunz-Jenkins, L. Foster, and E. Li. 1990. Cloning and expression of a membrane antigen of *Entamoeba histolytica* possessing multiple tandem repeats. *Proc. Natl. Acad. Sci. USA* **87**:4976-4980.
10. Stanley, S. L., Jr., T. F. H. G. Jackson, S. L. Reed, J. Calderon,

- C. Kunz-Jenkins, V. Gathiram, and E. Li. 1991. Serodiagnosis of invasive amebiasis using a recombinant *Entamoeba histolytica* protein. *JAMA* **266**:1984–1986.
11. 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.
12. Tonghai, Z., G. Youqiang, G. Xin, Z. Qicheng, L. Yuzheng, X. Buomin, W. Deen, and W. Juzhong. 1988. Seroepidemiological study on amoebiasis. *Chin. J. Epidemiol.* **9**:217–219.
13. Walsh, J. 1988. Prevalence of *Entamoeba histolytica* infection, p. 93–105. *In* J. I. Ravdin (ed.), *Amebiasis, human infection by Entamoeba histolytica*. John Wiley & Sons, Inc., New York.