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Molecular and Cellular Characterization of the 29-Kilodalton Peripheral Membrane Protein of *Entamoeba histolytica*: Differentiation between Pathogenic and Nonpathogenic Isolates

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To further characterize the 29-kDa surface antigen of *Entamoeba histolytica*, we analyzed the complete nucleotide sequence and compared the immunoreactivity of this antigen in pathogenic and nonpathogenic strains. Five cDNA clones (one 1.0-kb full-length clone, designated p13, and four partial-length clones) encoding the antigen were analyzed for allelic variation. Comparison of the nucleotide sequences revealed several single-nucleotide substitutions in all five cDNAs, two of which resulted in amino acid differences. Localization of the antigen to the amebic surface in a previous report (B. E. Torian, B. M. Flores, V. L. Stroeher, F. S. Hagen, and W. E. Stamm, Proc. Natl. Acad. Sci. USA 87:6358–6362, 1990) was corroborated by transmission electron microscopy showing colloidal gold particles on the surface of the trophozoites. Computer analysis of the deduced amino acid sequence predicted that the protein encoded by p13 was a hydrophilic peripheral membrane protein, and these data were confirmed by a Triton X-114 membrane extraction showing the presence of the 29-kDa antigen primarily in the aqueous phase of the detergent partition. Monoclonal antibodies to a fusion peptide differentiated between pathogenic and nonpathogenic clinical strains of *E. histolytica* in immunoblots. Although no immunoreactive epitopes were detected on nonpathogenic strains, Northern (RNA) analysis and DNA-DNA hybridization with a 700-bp cDNA probe demonstrated that mRNA and the gene encoding the 29-kDa surface antigen were present in both pathogenic and nonpathogenic clinical isolates.

Entamoeba histolytica infects 10% of the world's population and causes significant worldwide morbidity and mortality (31). Detailed structural and functional analysis of surface antigens which have genetic variability (6, 13, 25, 26) are necessary for potential vaccine development and to gain an understanding of host-parasite interaction. Edman et al. (6) identified and cloned a surface antigen of *E. histolytica* which was demonstrated to be a variable antigen and to be immunodominant on the basis of immunoblot reactivity of 73% of immune sera from patients with an amebic liver abscess. The antigen was unusually rich in tyrosine and asparagine residues. Similarly, two groups have sequenced individual clones of the *N*-acetyl-galactosamine-inhibitable lectin heavy chain from different axenically cultivated amebic strains (13, 25). This antigen is responsible for the initial attachment of *E. histolytica* to target tissue. Studies with a panel of monoclonal antibodies to the lectin suggest that there are distinct epitopes on pathogenic and nonpathogenic strains (15).

Zymodeme classification, based on the electrophoretic mobility of four glycolytic pathway enzymes, has been used to assign pathogenic or nonpathogenic status to clinical isolates of *E. histolytica* (18, 19). Controversy remains over whether isolates divided into distinct pathogenic and nonpathogenic zymodemes are stable or whether zymodeme switching from nonpathogenic to pathogenic zymodemes can occur. Although there have been reports of zymodeme switching during the process of amebic axenation (1, 14), epidemiological evidence suggests that this is a rare phenomenon (19). To date, 21 zymodemes have been reported and the majority of clinical isolates of *E. histolytica* fall into three zymodeme classifications: I, II, and III. The molecular and immunological characterization of pathogenic and nonpathogenic strains of ameba which possess antigens of similar molecular mass but which have subtle structural or immunological alterations may lead to an understanding of the function of this antigen(s).

In a previous report (27), we localized a 29-kDa antigen to the surface of the ameba and reported the nucleotide sequence of a partial cDNA clone. The antigen possessed a region within an 18-amino-acid segment which was unusually cysteine rich. In this report, using four partial cDNA clones and one full cDNA clone, we demonstrate that the cDNAs possessed different allelic types. The antigen was classified as a peripheral membrane protein on the basis of Triton X-114 partitioning which was followed by radioimmunoprecipitation. These data were corroborated by transmission electron microscopy and computer analysis of the deduced amino acid sequence. In addition, we showed that pathogenic and nonpathogenic *E. histolytica* clinical isolates could be differentiated on the basis of immunoblot reactivity with monoclonal antibodies. Further understanding of the structure and processing of this surface antigen may provide clues to functional or regulatory differences in pathogenic

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and nonpathogenic strains of *E. histolytica* and may also prove useful diagnostically and therapeutically.

MATERIALS AND METHODS

Strains, amebic culture, and maintenance. Axenic strains of *E. histolytica*, HM-1:IMSS (ATCC 30459), H-302:NIH (ATCC 30885), and H-303:NIH (ATCC 30887), were cultivated in Diamond's TYI-S-33 medium (5) supplemented with 15% bovine serum (Biofluids, Inc., Newton, Mass.). Amebae were harvested in mid-log phase and washed three times in ice-cold phosphate-buffered saline (PBS; 0.01 M, pH 7.6), and the final pellet was used immediately. Clinical isolates were cultured directly from stool or liver abscess into Robinson's medium (17), purified from medium components by Percoll gradient centrifugation, and then washed three times in PBS, which effectively eliminates 99.9% of surface-associated bacteria (16). The washed trophozoites were resuspended in PBS containing the following proteinase inhibitors: 1 mM (each) tosyl-lysyl-chloromethyl ketone, *N*-ethylmaleimide, EDTA, and phenylmethylsulfonyl fluoride (freshly prepared) and 100 μM E-64 [*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane]. These amebae were used immediately or frozen at −70°C for no longer than 2 weeks prior to use. Clinical pathogenic isolates tested included the following: SD 4, SD 53, SD 92, SD 135, SD 136, FAT 957, FAT 967, FAT 1014 (zymodeme II), and SAW 1519 (zymodeme XIV). Clinical nonpathogenic isolates used included the following: SD 11, SD 107, SD 130, SD 137, SD 138, SD 139, SD 147, FAT 973, FAT 1010 (zymodeme I), REF 291, and SAW 1734 (zymodeme III). SAW isolates were a kind gift from P. Sargeaunt, London School of Hygiene and Tropical Medicine, and FAT isolates were a gift from T. Jackson (Research Institute for Diseases in a Tropical Environment, Durban, Republic of South Africa).

Library screening and DNA sequencing. cDNA library construction, screening, and subcloning were carried out as previously described (27). Double-stranded sequencing of *E. histolytica* cDNA inserts p6, p16, p18 (partial clones), and p13 (full-length clone) was performed by the dideoxy chain termination method with the Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, Ohio) as previously described (27). Sequencing of both DNA strands was accomplished by using custom oligonucleotide primers (Core Laboratory Facility, Louisiana State University Medical Center, New Orleans, La.) and sequencing of subcloned *Hin*fI-digested fragments of purified p13 cDNA in the plasmid vector pGEM-4 (Promega, Madison, Wis.). Sequence analysis was performed by using the PC/GENE suite of programs from IntelliGenetics. Similarity searches were conducted on the nucleotide sequence of p13 or the largest open reading frame of p13 with FSTNSCAN or FSTPSCAN programs by using *k*-tuple values of 4 and 1, respectively, and EMBL (release 21) or Swiss Protein (release 13) data bases, respectively. The FSTNSCAN and FSTPSCAN programs are based on the FASTN algorithm of Lipman and Pearson (11). The nucleotide sequence of clone p47 has been described in a previous report (27).

DNA hybridization and Northern (RNA) analysis. DNA was extracted for DNA hybridization by the method of Kafatos et al. (8). Briefly, cell lysates containing 5×10^5 trophozoites were treated with 0.4 M Tris-Cl, pH 8.0, containing 0.1 M EDTA, 1% sodium dodecyl sulfate (SDS), and proteinase K (200 μg/ml) for 1 h at 55°C. After extraction with phenol and chloroform, the aqueous supernatant was treated with NaOH (0.3 M, final concentration) for 1 h at 65°C. Ammonium acetate was added to a final concentration of 1 M, and then twofold serial dilutions were made in 1 M ammonium acetate and applied by vacuum to nylon membranes (Hybond-N; Amersham, Arlington Heights, Ill.) with the Hybri-Slot manifold apparatus (Bethesda Research Laboratories). The DNA was hybridized with ^{32}P-labeled 700-bp partial cDNA (clone p47; 10^6 cpm/ml; specific activity, 10^7 cpm/μg).

RNA was isolated from fresh trophozoites by the method of Chomczynski and Sacchi (4). Ten micrograms of total RNA per lane was electrophoresed on a 1% agarose–2.2 M formaldehyde gel in morpholinepropanesulfonic acid (MOPS) buffer (12) and transferred (20) to GeneScreen Plus nylon membranes (NEN Research Products, Boston, Mass.). Hybridization was performed in Hybrisol I containing 50% formamide (Oncor, Gaithersburg, Md.) at 42°C by using the ^{32}P-labeled p47 cDNA.

Monoclonal antibodies, SDS-gel electrophoresis, and immunoblotting. The production of murine monoclonal antibodies specific for the 29-kDa protein has been previously described (27). Twenty-four independent monoclonal antibody lines were isolated from the fusion: 14 of the monoclonal antibodies immunoblotted the antigen, while 10 of the monoclonal antibodies immunoprecipitated the antigen. Polyxenic amebic lysates were prepared as previously described (2). The lysates (2×10^5 amebae per lane or 5×10^5 amebae per lane) were electrophoresed on SDS–10% polyacrylamide gels under reducing conditions (10) and transferred to nitrocellulose membranes (30). Immunoblotting was performed as previously described by using protein A-purified monoclonal antibodies specific for the 29-kDa antigen (final concentration, 2 μg/ml) (27).

Triton X-114 detergent partitioning and immunoprecipitation. *E. histolytica* trophozoites were labeled with [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-label; ICN, Irvine, Calif.), harvested, and washed three times with PBS as previously described (28). Antigen from 10^6 trophozoites was partitioned with 0.75% Triton X-114 by the method of Bordier (3). After partitioning, 2 ml of solubilization buffer (28) was added to the detergent and aqueous phases, and radioimmunoprecipitations were performed with purified monoclonal antibody FP-28 as previously described (27, 28). The samples were electrophoresed on an SDS–10% polyacrylamide gel, dried in vacuo, and subjected to fluorography. The immunoprecipitated 29-kDa band in the aqueous and detergent phases was scanned on a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Electron microscopy. Freshly harvested *E. histolytica* trophozoites were washed, resuspended in 1 ml of PBS, and then incubated for 1 h on ice with 500 μg of monoclonal antibody FP-28 or a matched isotype (to FP-28) of purified monoclonal antibody specific for *Pneumocystis carinii*. Excess antibody was removed by four washes with ice-cold PBS, which was followed by incubation with electron microscopy grade colloidal gold-conjugated goat anti-mouse immunoglobulin G (10-nm-diameter gold particles; Zymed Inc.) for 1 h on ice. Membrane integrity of trophozoites was assessed by Trypan blue exclusion prior to and after incubation with antibody and with conjugate. Greater than 99% of the organisms excluded the dye. The cells were washed gently four times with ice-cold PBS and then fixed in 4.0% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 h. The fixative was dripped onto the pelleted cells, and the pellet was broken up into small pieces (approximately 0.5 mm^3) after 30 min. The fixed cells were washed three times in 0.1 M cacodylate buffer, postfixed with 0.1% osmium

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1                                   CAAAATTTC AATATTATA ATTATTTTA AGTTGTATT TTTTCATTA AGTTATAGT TTTGAACAA AATTATGAA

73  ATTGTTCAT CAAAAAAGA GTATTAACA GAAGGACAA AAGATAGCA TTAAGGAAT AGACAATTT GTAATTAAT TATTATAAG AATTTAGCA AAAGAACAA

1   M  K  R  R  K  I  I  K  Y  D  S  P  S  Y  D  D  I  A  D  K  Q  R  N  E  L  S  N  V  Y  N  E  I  L
172 ATGAAGAGA AGAAAAATT ATTAAATAT GATTCACCA TCATATGAT GACATTGCT GATAAACAA CGAAATGAG TTAAGTAAT GTTTATAAT GAAATTCTT

34  Q  K  K  L  R  E  K  G  R  M  E  V  M  K  E  I  K  A  K  E  C  G  E  K  E  C  C  K  E  C  C  C  P
271 CAAAAGAAA TTAAGAGAA AAAGCAAGA ATGGAAGTT ATGAAAGAA ATAAAAGCA AAAGAATGT CAAGAGAAAGAATGTTGT AAAGAATGT TGTTGTCCA

67  R  I  K  A  F  K  K  F  I  N  T  F  E  K  A  Q  I  G  K  E  A  P  P  F  K  A  P  A  Y  C  P  C  G
370 AGAATAAAA GCATTTAAG AAATTTATA AACACATTT GAAAAAGCA CAAATTGGA AAAGAAGCA CCAGAATTT AAAGCACCA GCATATTGT CCATGTGGT

100 S  I  K  E  I  D  I  N  E  Y  R  G  K  Y  V  V  L  L  F  Y  P  L  D  W  T  F  V  C  P  T  E  M  I
469 TCAATCAAA GAGATTGAT ATTAATGAA TATAGAGGA AAATATGTT GTATTGTTG TTTTATCCA TTGGATTGG ACATTTGTT TGTCCAACA GAAATGATT

133 G  Y  S  E  L  A  G  Q  L  K  E  I  D  C  E  V  I  G  V  S  V  D  S  V  Y  C  H  Q  A  W  C  E  A
568 GGATATAGT GAACTTGCA GGACAATTG AAAGAAATC GATTGTGAA GTTATTGGA GTGAGTGTA GATTCAGTT TATTGTCAT CAAGCATGG TGTGAAGCA

166 D  K  S  K  G  G  V  G  K  L  T  F  P  L  V  S  D  I  K  R  C  I  S  I  K  Y  G  M  L  N  V  E  A
667 GATAAAAGT AAAGGAGGA GTAGGAAAG TTGACATTC CCATTAGTA TCAGATATT AAGAGATGC ATTTCTATC AAATATGGA ATGTTAAAT GTTGAAGCA

199 G  I  A  R  R  G  Y  V  I  I  D  D  K  G  K  V  R  Y  I  Q  M  N  D  D  G  I  G  R  S  T  E  E  T
766 GGAATTGCA AGAAGAGGA TATGTCATC ATTGATGAT AAAGGAAAA GTAAGATAC ATTCAAATG AATGATGAT GGAATTGGA AGATCAACG GAAGAAACA

232 I  R  I  V  K  A  I  Q  F  S  D  E  H  G  A  V  C  P  L  N  W  K  P  G  K  D  T  I  E  P  T  P  D
865 ATCAGAATA GTTAAAGCA ATTCAATTC AGTGATGAA CATGGAGCA GTTTGTCCA CTCAATTGG AAACCAGGC AAAGACACC ATTGAACCA ACACCAGAT

265 G  I  K  K  Y  L  T  A  H  end
964 GGAATTAAG AAATATTTA ACAGCACAT TAAAACAAA TAAGATAAT TT
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FIG. 1. Nucleotide and deduced amino acid sequence of p13 cDNA, which encodes a 29-kDa *E. histolytica* protein. The sequence is 1,010 nucleotides in length with 171 untranslated nucleotides at the 5′ end and 17 untranslated nucleotides at the 3′ end. The ribosomal binding site (21) which coincides with the ATG translation initiation codon and the cysteine-rich tandem repeat are underlined. Numbers at left denote nucleotide and amino acid positions.

tetroxide in 0.1 M cacodylate buffer for 2 h, and then block stained with 0.05% aqueous uranyl acetate overnight. The cells were washed twice in deionized water and dehydrated with an ascending series of ethanol (50 to 100% ethanol, three times in 100%) and propylene oxide (three times). Infiltration was carried out in an ascending series of Epon-Araldite epoxy resin (Polybed; Polysciences) mixed with propylene oxide (25 to 75% resin) and 100% plastic (two times). Cells were transferred into fresh plastic in flat molds and polymerized in a 60°C oven. Gold ultrathin sections were obtained on a Reichardt OMu3 microtome with a diamond knife. Sections were examined on a Philips CM10 electron microscope at an accelerating voltage of 60 kV.

Nucleotide sequence accession number. The nucleotide sequence data for p13 have been assigned GenBank number M75858.

RESULTS

Sequence analysis. The nucleotide sequence was determined for three partial clones designated p6 (0.78 kb), p16 (0.8 kb), and p18 (0.71 kb) and one full-length clone designated p13 (1.0 kb) (Fig. 1, data shown for p13). The full sequence of p13 was 1,010 nucleotides in length and A-T rich (69%), revealing 700 inverted repeats of at least 5 bases in length. When analyzed with a 10-base minimum length, three inverted and three direct repeats were identified. Additionally, a 12-nucleotide cysteine-rich tandem repeat starting at position 340 was identified. When the nucleotide sequences of the three partial clones and that of p47 (27) were compared with the nucleotide sequence of p13, three single nucleotide substitutions (polymorphisms) resulting in amino acid substitutions in two positions were detected (Fig. 2), while other base changes represented nucleotide polymorphisms only.

A ribosomal binding site at nucleotide position 169 (21) which coincided with the ATG translation initiation codon of the single long open reading frame of p13 (Fig. 1) was identified. The protein encoded by p13 was composed of 273 amino acids (12% lysine, 9.8% isoleucine, 8.7% glutamic acid, and 5.1% cysteine) and had a predicted molecular mass of 31,168 Da, a pI of 8.0, multiple potential phosphorylation sites at serine residues, and one myristilation site (data not shown). The protein was hydrophilic in nature (Fig. 3) and showed no evidence of a transmembrane sequence. In addition, p13 was predicted by the method of Klein et al. (9) to be a peripheral membrane protein and not an integral membrane protein.

Triton X-114 partitioning. Triton X-114 phase separation was used to partition membrane proteins of *E. histolytica*. Following extraction, radioimmunoprecipitations with monoclonal antibody were performed on detergent and aqueous phases. Analysis of the fluorograph revealed that the 29-kDa antigen was present predominantly in the aqueous phase of

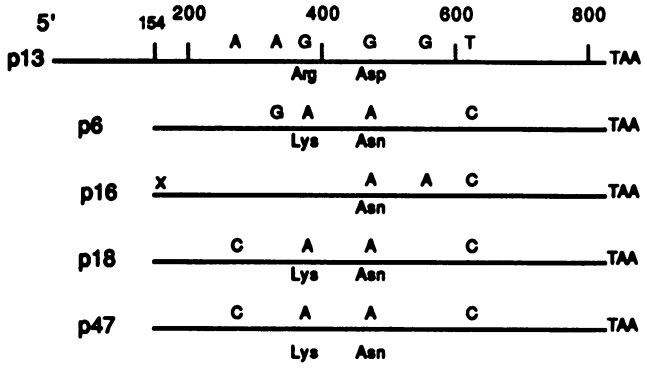

FIG. 2. Analysis of nucleotide substitutions in the cDNA sequences showing allelic variation. The cDNA sequences of the four partial clones were compared with that of p13 and showed identity with p13 from 154 bases downstream of the presumptive start codon. Letters denote nucleotide substitutions, and amino acid differences are indicated. X, deletion of 12 bases (AAA GAA TGT TGT) of the tandem repeat.

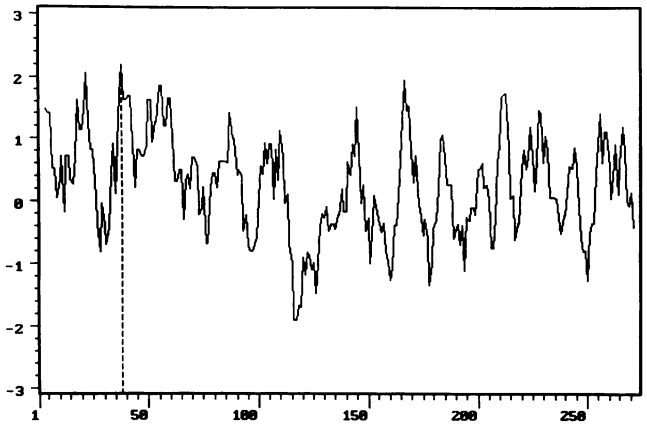

FIG. 3. Hydrophilicity analysis of deduced amino acid sequence of p13 showing a hydrophilic protein and a potential antigenic determinant from amino acids 35 to 40 (Lys-Lys-Leu-Arg-Glu-Lys).

the partition (Fig. 4, lane 5), and a faint band at 29 kDa could be seen in the radioimmunoprecipitation of the detergent phase. Scanning on a Phosphor Imager, more sensitive than a visual fluorographic examination, showed an approximate 10:1 ratio of beta emission from the 29-kDa band in the aqueous versus the detergent phase (aqueous phase, 8.2×10^5 cpm; detergent phase, 8.6×10^4 cpm).

Electron microscopy. To determine whether the 29-kDa antigen was on the outer surface, live trophozoites were reacted with monoclonal antibody followed by colloidal gold-labeled anti-mouse immunoglobulin G. Electron microscopic analysis demonstrated that the antigen was present on the external surface of the plasma membrane and appeared to be associated with the amebic glycocalyx rather than the plasma membrane. Gold label was usually localized in one cluster on the surface of the trophozoites (Fig. 5A). Colloidal gold particles were not detected on *E. histolytica* trophozoites when reacted with anti-*P. carinii* monoclonal antibody (Fig. 5B).

Detection of the 29-kDa surface antigen on clinical isolates. To determine the specificity of monoclonal antibodies for the native 29-kDa antigen, we tested amebic clinical isolates of *E. histolytica* by immunoblotting. The monoclonal antibodies reacted with the 29-kDa antigen on seven pathogenic isolates but not with nine nonpathogenic isolates tested (Table 1 and Fig. 6). When immunoblotting was performed with 2.5 times more nonpathogenic trophozoites (5×10^5 amebae per lane), the monoclonal antibodies failed to detect the 29-kDa antigen (data not shown). Because of the extremely sensitive nature of the antigen to degradation, only freshly harvested log-phase amebae or amebic lysates stored at −70°C for no more than 2 weeks were used (unpublished data).

Northern (RNA) analysis and DNA-DNA hybridizations. Northern analysis of total RNA isolated from two pathogenic and three nonpathogenic clinical isolates by using the 700-bp partial cDNA (p47) probe identified a single 1.05-kb band, demonstrating the presence of the 29-kDa antigen mRNA in both pathogenic and nonpathogenic isolates (Fig. 7). DNA extracted from eight clinical isolates of *E. histolytica* (four pathogenic and four nonpathogenic zymodeme isolates) was hybridized with the p47 probe. The probe hybridized with DNA from all isolates tested (Fig. 8, data shown for one pathogenic and one nonpathogenic zymodeme) but not with DNA isolated from associated bacteria (data not shown). However, with the same number of trophozoites from pathogenic or nonpathogenic isolates, there was a distinct difference in the intensity of hybridization to the probe, with more intense hybridization to DNA from the pathogenic isolates (Fig. 8B).

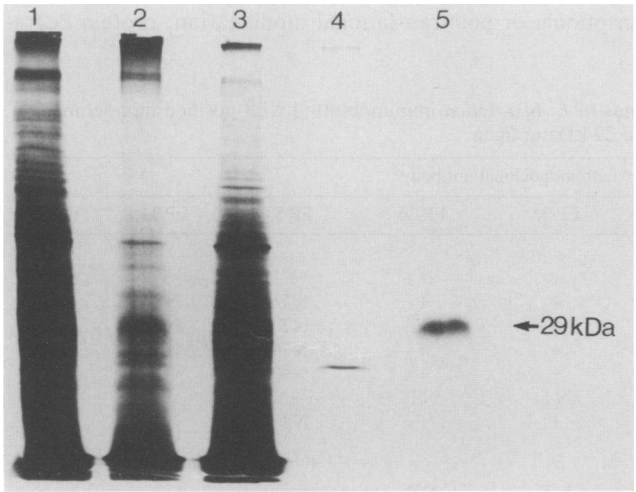

FIG. 4. Triton X-114 detergent partitioning of ^{35}S-labeled *E. histolytica* and subsequent radioimmunoprecipitation of integral membrane proteins (detergent phase) and soluble proteins (aqueous phase) by using monoclonal antibody (FP-28) specific for the 29-kDa antigen. Lane 1, whole-protein profile of ^{35}S-labeled *E. histolytica*; lane 2, whole profile of proteins partitioned in the detergent phase; lane 3, whole profile of proteins partitioned in the aqueous phase; lane 4, radioimmunoprecipitation of detergent phase; lane 5, radioimmunoprecipitation of the aqueous phase demonstrating the 29-kDa antigen.

DISCUSSION

In this report, we have characterized the 29-kDa antigen of *E. histolytica* with monoclonal antibodies which distinguish between pathogenic and nonpathogenic zymodeme classified amebic strains and isolates. The sequences of four partial cDNA clones and one full-length cDNA clone which encode for this antigen demonstrate a number of interclonal nucleotide substitutions probably generated from heterologous nuclear mRNA used to prepare the cDNA library, although a single cloned amebic isolate was used for mRNA isolation. The nucleotide substitutions were consistent among the five cDNA clones, suggesting that the substitutions did not result from cloning but in fact represent nucleotide polymorphisms. With other antigens, investigators have reported 1% variability in the amino acid sequence deduced from independently isolated cDNAs for the same gene from *E. histolytica* HM-1:IMSS isolates and inferred from the amino acid replacements even greater nucleotide variability (6). Analysis of the 29-kDa antigen cDNAs revealed that few repeats over 10 bases in length were found compared with the number of repeats found in DNA sequences of other *E. histolytica* antigens (22, 25). Because the sequence is A-T rich, it is prone to small repeats (both inverted and direct); however, the significance of the repeats is unknown with regard to the coded protein.

We previously reported a partial cDNA (p47) with a region relatively rich in cysteine and an overall cysteine content of 7.3% (27). Although the overall cysteine content of p13 was

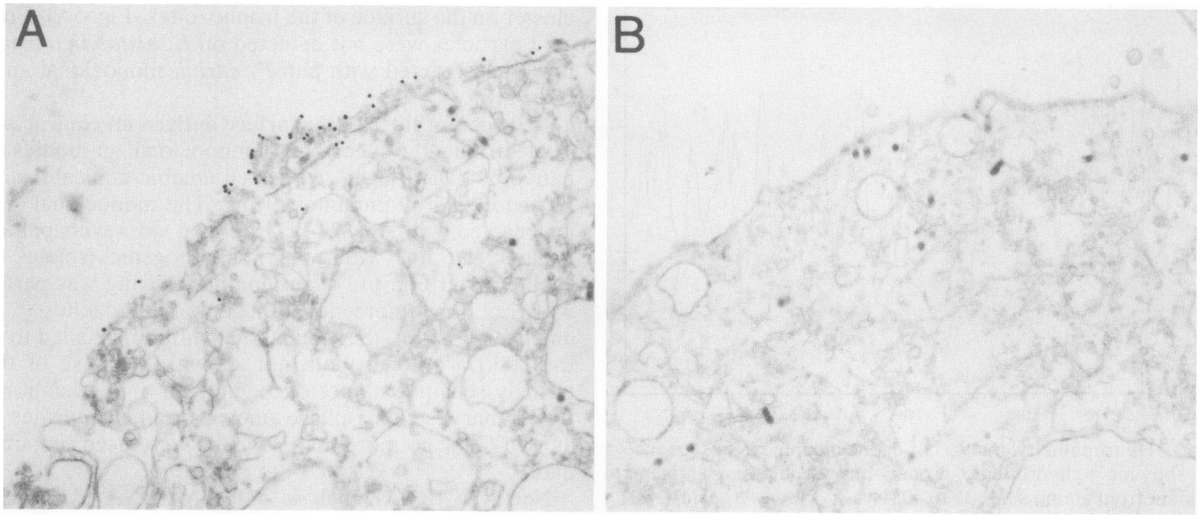

FIG. 5. Transmission electron microscopic localization of the 29-kDa antigen to the peripheral membrane of *E. histolytica*. Live trophozoites were incubated with monoclonal antibody and localization of antibodies was detected with 10-nm colloidal gold-conjugated goat anti-mouse immunoglobulin G. (A) Reactivity of monoclonal antibody FP-28 with live *E. histolytica* trophozoite demonstrating colloidal gold on the surface. (B) Reactivity of a matched isotype monoclonal antibody specific for *P. carinii* with live *E. histolytica* showing no staining with colloidal gold.

5.1%, a cysteine-rich region was identified and associated with a tandem repeat beginning at position 169. This tandem repeat coincides with the cysteine-rich region in p47 and was also present in p6 and p18 but absent in p16. The importance of thiol groups on the surface of the organism, or which are readily accessible, in providing a reducing environment for the survival of several enteric protozoan species including *E. histolytica* has been demonstrated by Gillin et al. (7).

Homology searches of both the nucleotide sequence and the deduced amino acid sequence of p13 revealed no significant similarity between this cDNA or protein and others. Interestingly, the greatest degree of identity found was between the P190 gene of *Plasmodium falciparum* (PFP190G1) which codes for a merazoite surface antigen precursor, a *P. falciparum* serine repeat RNA (PFSERA), the *exp-1* gene coding for malaria protein exp-1 (PFEXP1G), and the *P. falciparum* major merazoite surface antigen mRNA (PFGP 195A), each of which possessed 53 to 58% identity with the nucleotide sequence of the 29-kDa *E. histolytica* surface antigen.

The translated protein of p13 had a predicted molecular mass of 31,168 Da, while the native protein detected by immunoblotting and immunoprecipitation comigrated with the 29-kDa molecular mass marker. The difference of 2 kDa in the observed and predicted size may be due to posttranscriptional or posttranslational modification, protein degra-

TABLE 1. Reactivity of pathogenic and nonpathogenic clinical isolates of *E. histolytica* immunoblotted with purified monoclonal antibodies specific for the 29-kDa antigen

Strain	Reactivity[a] with monoclonal antibody:								
	FP-9	FP-14	FP-19	FP-29	FP-33	FP-36	FP-8	FP-11	FP-31
Pathogenic									
SD 4	+	+	+	+	+	+	+	+	+
SD 53	NT	+	+	+	+	+	NT	NT	NT
SD 92	+	NT	+	+	+	NT	NT	NT	NT
SD 135	NT	+	+	+	+	+	NT	NT	NT
SD 136	+	+	+	+	+	+	+	+	+
FAT 957	NT	+	NT	NT	NT	NT	+	+	+
FAT 1014	NT	+	+	+	+	+	NT	NT	NT
Nonpathogenic									
SD 107	NT	NT	–	–	NT	NT	–	–	–
SD 130	–	–	–	–	–	–	NT	NT	NT
SD 137	–	NT	–	–	–	NT	NT	NT	NT
SD 138	–	–	NT	NT	–	–	NT	NT	NT
SD 139	NT	–	NT	NT	NT	–	NT	NT	NT
FAT 973	–	–	–	–	–	–	NT	NT	NT
FAT 1010	–	–	–	–	–	–	NT	NT	NT
REF 291	–	–	–	–	–	–	–	–	–
SAW 1734	NT	–	NT	NT	NT	NT	–	–	–

[a] +, reactivity with 29-kDa antigen; –, no reactivity with 29-kDa antigen; NT, not tested.

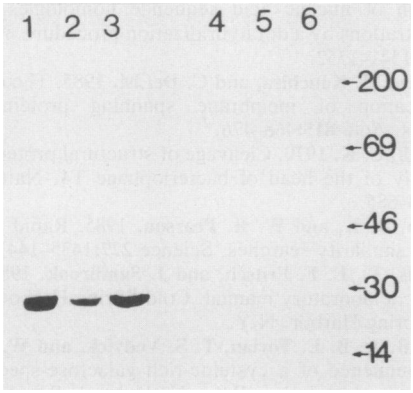

FIG. 6. Immunoblot reactivity of protein A-purified monoclonal antibody FP-14 to representative pathogenic and nonpathogenic clinical isolates of *E. histolytica*. Lysates containing 2×10^5 amebae per lane were loaded, electrophoresed under reducing conditions on SDS–10% polyacrylamide gels, and transferred to nitrocellulose membranes. The blots were incubated with 20 μg of monoclonal antibody and detected with horseradish peroxidase-conjugated protein A, 4-chloro-1-naphthol, and H_2O_2. Lanes 1 to 3, pathogenic isolates; lanes 4 to 6, nonpathogenic isolates.

dation, or the inaccuracy of gel electrophoresis for determination of molecular mass.

In a previous report, we detailed the surface nature of the antigen (27). Sequence analysis of p13 and transmission electron microscopy corroborate our previous findings. The localization of colloidal gold particles on the surface of the trophozoites appeared to show peripheral membrane association rather than integral association with the plasma membrane. The studies showed clustered distribution of colloidal gold particles over the external amebic surface. These findings contrast with previous immunofluorescence analysis of live and formalin-fixed trophozoites which showed uniform staining over the entire surface of the

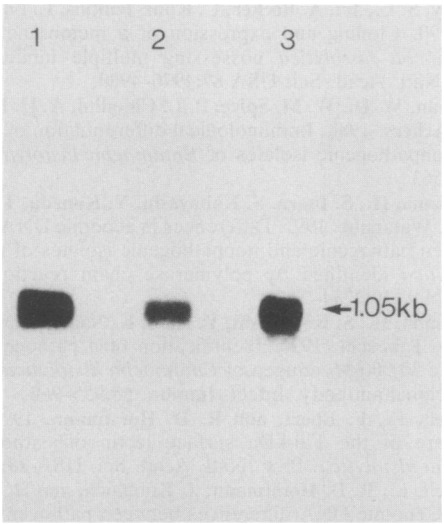

FIG. 7. Northern analysis of mRNA isolated from representative pathogenic and nonpathogenic clinical isolates of *E. histolytica* probed with ^{32}P-labeled purified p47. Lane 1, *E. histolytica* SD 4, zymodeme II (pathogenic); lane 2, *E. histolytica* SAW 1734, zymodeme III (nonpathogenic); lane 3, *E. histolytica* SD 130, zymodeme III (nonpathogenic).

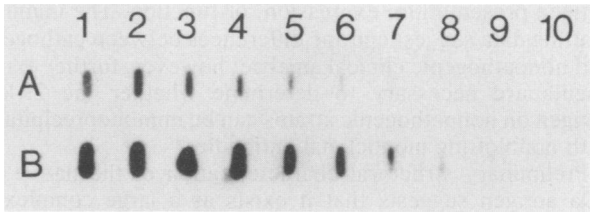

FIG. 8. DNA-DNA hybridization analysis of representative pathogenic and nonpathogenic *E. histolytica* zymodemes by using the ^{32}P-labeled p47 cDNA probe. (A) Strain SD 107 (nonpathogenic zymodeme I); (B) strain FAT 967 (pathogenic zymodeme II). Lane 1, DNA extracted from amebic lysates containing 2×10^5 trophozoites; lanes 2 to 10, twofold serial dilutions of the DNA extracted from 2×10^5 trophozoites.

organisms (27). The clustering observed with colloidal gold may have been the result of extended incubation times. Although amebae purportedly do not cap and shed antibody at the incubation temperatures used for this procedure, some membrane fluidity and potential receptor-surface antigen clustering may have occurred.

The 29-kDa antigen was found to be extremely labile to degradation. Whether this phenomenon indicates autoproteolysis or rapid degradation by specific *E. histolytica* proteases has not been elucidated. Amebic lysates from clinical isolates or axenic strains had to be utilized in experiments immediately or within 2 weeks after storage at −70°C or the antigen was not found intact by immunoblotting. Interestingly, this phenomenon was seen in a previous study in which one pathogenic strain (FAT 1014) did not react with monoclonal antibody FP-33 (27). Current experiments using fresh clinical isolates showed reactivity to this monoclonal antibody. Additionally, we also reported weak reactivity to two nonpathogenic isolates by two monoclonal antibody-bearing ascites (27). When purified monoclonal antibodies were used (current study), reactivity to nonpathogenic isolates was not detected even when more nonpathogenic trophozoites were tested per blot.

Although no 29-kDa immunoreactive protein could be detected on nonpathogenic strains with nine monoclonal antibodies, Northern analysis as well as DNA-DNA slot blot hybridization with the 700-bp probe demonstrated that the message and gene were likely present in nonpathogenic zymodeme isolates. The lack of immunoreactivity in nonpathogenic isolates could be explained by amino acid differences. The epitope differences between nonpathogenic and pathogenic isolates could make this antigen a candidate for an antibody-based diagnostic test. Similarly, a battery of monoclonal antibodies to different epitopes of the attachment lectin which can detect the antigen on pathogenic and nonpathogenic clinical isolates as well as differentiate pathogenic and nonpathogenic strains were generated (15). Additional support for these observations is provided by reports of monoclonal antibodies which selectively react with pathogenic zymodeme isolates (1, 2, 23, 24, 29). However, these reports are based on immunofluorescence or immunoblotting alone.

Edman et al. (6) analyzed the nucleotide sequence of genomic DNA encoding a 125-kDa antigen in pathogenic and nonpathogenic clinical isolates and demonstrated a 10% difference in the nucleotide sequence and a 12% difference in the amino acid sequence between pathogenic and nonpathogenic strains. Differences in nucleotide sequence and commensurate amino acid sequence may result in alterations in

antigen presentation, expression, or function. The immunoblotting data suggest epitope differences between pathogenic and nonpathogenic clinical amebae; however, further experiments are necessary to determine whether the 29-kDa antigen on nonpathogenic strains can be immunoprecipitated with nonblotting monoclonal antibodies.

Preliminary structural characterization of the native 29-kDa antigen suggests that it exists as a large complex of greater than 240 kDa. Work is presently underway to determine if the protein is present on nonpathogenic isolates and to evaluate the immunoreactivity of the monoclonal antibodies with a variety of zymodemes to assess the diagnostic potential of the antibodies. Current efforts are also directed at sequencing genomic DNA as well as the gene from nonpathogenic isolates and obtaining a full structural characterization of the antigen in addition to testing for potential use as a vaccinogen.

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ADDENDUM

Since submission of the manuscript, Tachibana et al. (23a) have reported the nucleotide sequence of a 0.7-kb cDNA with complete homology with the deduced amino acid sequence of the partial cDNA sequence of the 29-kDa antigen we previously published. In addition, they demonstrated differences between pathogenic and nonpathogenic strains on the basis of restriction endonuclease digestion of polymerase chain reaction-amplified DNA coding for the antigen.

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