Protection of Gerbils from Amebic Liver Abscess by Immunization with Recombinant *Entamoeba histolytica* 29-Kilodalton Antigen

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The goal of our study was to obtain a highly conserved *Entamoeba histolytica* recombinant antigen for study as a subunit amebiasis vaccine. We screened a Uni-Zap cDNA library of *E. histolytica* (strain HM1:IMSS) with human immune sera and isolated a dominant 804-bp cDNA clone. A 33-kDa fusion protein expressed from the cDNA clone was determined by monoclonal antibody binding, DNA hybridization, and nucleotide sequence to be the complete *E. histolytica* 29-kDa antigen. Serum antibodies to the recombinant protein were detected by enzyme-linked immunosorbent assay in 80% of subjects from Egypt and South Africa with amebic liver abscess. Similar results were found with the native 29-kDa protein. Native and recombinant 29-kDa antigens induced proliferation of lymphocytes harvested from patients with amebic liver abscess ($P < 0.01$ compared with controls). Intraperitoneal immunization of gerbils with the recombinant fusion protein (10 μ g) with Titermax adjuvant elicited an antigen-specific serum immunoglobulin G antibody response and was partially protective (54%) against intrahepatic challenge with 5×10^5 virulent axenic trophozoites (strain HM1:IMSS). In summary, the recombinant form of the *E. histolytica* 29-kDa antigen demonstrated serologic specificity for amebic liver abscess, exhibited conserved T-cell epitopes, and was effective as a subunit vaccine in an experimental animal model of amebic liver abscess.

Entamoeba histolytica is a widespread enteric protozoan that causes significant worldwide morbidity and mortality (23). Invasive amebiasis manifests as colitis or liver abscess (24); however, in many areas of the world the greatest disease burden is due to hepatic amebiasis (23, 24). Currently, no vaccine or chemoprophylaxis is available to protect individuals residing in or traveling to areas where *E. histolytica* is endemic.

Highly conserved surface membrane antigens that induce serum antibody or cell-mediated immune responses hold promise for being protective against invasion of *E. histolytica* trophozoites, especially in the liver. Recently, several surface antigens have been identified, including the 260-kDa galactose-inhibitable adherence lectin (10, 11), a 125-kDa antigen (5), a serine-rich protein (18), and the 29-kDa antigen (20-22). The adherence lectin has been demonstrated to be a highly conserved *E. histolytica* antigen, recognized by serum antibodies in patients with invasive amebiasis worldwide (12). The 260-kDa adherence protein plays an important role in amebic adhesion and contact-dependent cytolysis (10, 11). The 125-kDa antigen, identified and cloned by Edman et al. (5), was demonstrated to elicit an antibody response on the basis of immunoblot reactivity of 73% of immune sera from patients with amebic liver abscess. The serine-rich *E. histolytica* protein is a 47- to 52-kDa surface molecule. Greater than 80% of patients with amebic liver abscess have serum antibodies to the recombinant serine-rich protein (18).

The 29-kDa protein and a 700-bp cDNA encoding a portion of the antigen were first isolated by Torian et al. (22) from the

E. histolytica H-302:NIH strain. Subsequently, the same antigen, designated 30 kDa, was identified in the more virulent HM1:IMSS axenic strain (20). The 714-bp HM1:IMSS cDNA reported by Tachibana et al. (20) was highly homologous with that reported by Torian et al. (22). The genomic DNA sequence of the 29-kDa antigen contains CATTAAA, a likely TATA box, ATCA, a transcription start site, and a full coding region (1). Molecular and cellular characterizations indicate that the 29-kDa antigen is a peripheral membrane protein which possesses an unusually cysteine-rich 18-amino-acid segment, although there is controversy regarding the cellular location of the antigen (1, 21). The 29-kDa protein has epitopes unique to pathogenic *E. histolytica* isolates, since they were not detected in nonpathogenic isolates (*Entamoeba dispar*) by monoclonal antibody probes (13). Enzyme-linked immunosorbent assay (ELISA) studies using native 29-kDa antigen demonstrated that serum antibodies to the 29-kDa protein were present in 79 to 87% of patients with amebic liver abscess (3).

By screening an HM1:IMSS cDNA library with immune sera obtained from patients with amebic liver abscess, we isolated an 804-bp cDNA clone encoding the entire 29-kDa protein. A recombinant fusion protein was expressed and purified; antigenicity was determined by serum antibody recognition and T-cell proliferation. To evaluate the promise of this recombinant protein for use in an amebiasis subunit vaccine, immunogenicity and vaccine efficacy were determined in the gerbil model of amebic liver abscess.

MATERIALS AND METHODS

Construction of an *E. histolytica* cDNA library from strain HM1:IMSS. Total RNA was isolated from axenic *E. histolytica* trophozoites (strain HM1:IMSS) at the mid-logarithmic phase of growth by the method described by Chomczynski

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and Sacchi (3). mRNA was obtained by passing the total RNA through poly(A) Quick Columns (Stratagene, LaJolla, Calif.). cDNA was constructed by using a mixture of calf thymus random primer and oligo(dT) primers. cDNAs greater than 400 bp in size were selected for ligation to Uni-ZAP (Stratagene) and packaged with Stratagene's Gigapak II Gold. The titer of the amplified cDNA library was 2.0×10^{10} /ml (represents 10^6 PFU).

Screening of the cDNA library with human immune sera and sequencing of the p48 clone. Recombinant phage (10^6) of the HM1:IMSS cDNA library were screened with pooled sera from convalescent patients with amebic liver abscess at a 1:100 dilution. An alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG) antibody was used to visualize positive plaques with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and Nitro Blue Tetrazolium. All positive cDNA clones were subjected to three sequential screenings with immune sera and purified as single clones after the third screening. The DNA inserts of the positive clones were analyzed after automatic excision of cDNAs from Uni-ZAP to pBluescript SK- (Stratagene) according to the manufacturer's instructions.

Double-stranded sequencing of p48 was performed by using the dideoxy chain termination method (16) with M13 universal and reverse primers and an Auto-Read sequencing kit (Pharmacia, Alameda, Calif.). Sequencing gels were analyzed by an Automated Laser Fluorescent automatic sequencer (Pharmacia).

Identification of cDNA clones encoding the *E. histolytica* 29-kDa antigen. pBluescript colonies containing inserts were grown in the XL1-blue strain, and protein production was induced with isopropyl- β -D-thiogalactopyranoside (IPTG) (2 mM). Cells were lysed with 1% sodium dodecyl sulfate (SDS), and lysate was spotted on a nitrocellulose membrane for incubation with an anti-29-kDa-protein IgG monoclonal antibody (FP14) (8). Positive lysates were identified by horseradish peroxidase-linked protein A and developed with 4-chloro-1-naphthol and H_2O_2 .

DNA dot blotting was carried out by spotting denatured DNA plasmids on a nitrocellulose membrane. The 700-bp 29-kDa-antigen-specific probes were labeled by using the Genius system (Boehringer Mannheim, Indianapolis, Ind.); hybridization with the DNA plasmid was performed according to the manufacturer's recommendations (9).

Expression and purification of the 29-kDa recombinant protein. The gene for the 29-kDa protein was released from the p48 clone by digestion with the restriction enzymes *Bam*HI and *Xho*I. The gene was sequentially subcloned in frame into a pRSET expression vector. The recombinant constructs were transformed into *Escherichia coli* BL21(DE3) (Novagen, Madison, Wis.) containing the gene for T7 polymerase. Recombinant protein was expressed by induction with 0.5 mM IPTG at the mid-log phase of bacterial growth with cultivation for an additional 2 h.

The recombinant fusion protein containing a 5' sequence of six histidine residues was purified by immobilized metal affinity chromatography. Briefly, the cells were harvested by centrifugation at $4,000 \times g$ for 10 min. The cell pellet was washed once with Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and suspended in the buffer for storage at -70°C . To lyse the bacterial cells, the suspension was thawed at 37°C and lysozyme was added to a final concentration of 100 $\mu\text{g}/\text{ml}$. The DNA was digested with DNase (1 U/ml), and soluble protein was separated by centrifugation at $16,000 \times g$ for 20 min. The supernatant was applied to a Probond column (Invitrogen, San Diego, Calif.) which was equilibrated with a binding buffer containing 20 mM Tris-HCl (pH 7.9) in 0.5 M NaCl (buffer A) with 5 mM imidazole. The column was washed with the binding buffer and with a wash buffer containing 33 mM imidazole in buffer A. The bound proteins were eluted with buffer A containing 300 mM imidazole.

The recombinant 29-kDa protein was further purified from the eluate by immunoaffinity chromatography with an anti-29-kDa-protein monoclonal antibody (22). The purity and specificity of the final recombinant fusion protein was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The yield for the recombinant protein was 200 $\mu\text{g}/\text{liter}$ of bacterial culture.

ELISA for serum antiamebic antibodies and studies of lymphocyte proliferation. ELISA was performed as described previously (17). Ninety-six-well microtiter plates (flat-bottomed, polystyrene ELISA plates; Costar, Cambridge, Mass.) were coated by inoculation at 4°C overnight with 100 μl of 0.1 M carbonate buffer (pH 9.8) containing 125 ng of recombinant antigen or 100 ng of the native 29-kDa protein. Plates were washed twice with phosphate-buffered saline (PBS)-Tween-1% bovine serum albumin (BSA) and blocked with 1% BSA. The blocking reagent was removed, and human serum diluted to 1:500 (in the case of recombinant protein) or 1:100 (in the case of native 29-kDa protein) in PBS-Tween-1% BSA was added for overnight incubation at 4°C . The optimal amount of protein and dilution of serum to be used in the ELISA were determined by checkerboard titration. The wells were washed four times with PBS-Tween and incubated with 100 μl of a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-human IgG antibody (Bio-Rad, Hercules, Calif.) for 2 h at room temperature, and the peroxidase-specific color reaction was developed in the dark for 30 to 90 min; A_{492} was measured. Results were corrected for specific background by subtracting the optical densities from those of paired wells not coated with protein but otherwise exposed to the identical procedure. Each serum sample was tested in duplicate.

Lymphocyte proliferation was determined by [^3H]thymidine uptake as reported previously (15). Concanavalin A (20 $\mu\text{g}/\text{ml}$), whole soluble amebic anti-

1	G	GCA	CGA	GAA	ACA	TCT	CAT	CAT	CAC	CAC	AAT	CTC	ATC	AAT	TCA	ATC	46
47	AGT	CAA	ATT	TCT	TGC	AAT	CAA	CAA	AAA	GAG	TGT	TGT	AAA	AAA	GAA	TGT	94
1		M	S	C	N	S	C	K	E	C	C	K	E	C	K	E	14
95	CAA	GAG	AAA	GAA	TGT	TGT	AAA	GAA	TGT	TGT	TGT	CCA	AGA	ATA	AAA	GCA	142
15	Q	E	K	E	C	C	K	E	C	C	P	R	R	I	K	A	30
143	TTT	AAG	AAA	TTT	ATA	AAC	ACA	TTT	GAA	AAA	GCA	CAA	ATT	GGA	AAA	GAA	190
31	F	K	K	F	I	N	T	F	E	K	A	Q	I	G	K	E	46
191	GCA	CCA	GAA	TTT	AAA	GCA	CCA	GCA	TAT	TGT	CCA	TGT	GGT	TCA	ATC	AAA	238
47	A	P	E	F	K	A	P	A	Y	I	D	C	G	S	I	K	62
239	GAG	ATT	GAT	ATT	AAT	GAA	TAT	AAA	GGA	AAA	TAT	GTT	GTA	TTG	TTG	TTT	286
63	E	I	D	I	N	E	Y	K	G	A	Y	V	L	L	F		78
287	TAT	CCA	TTG	GAT	TGG	ACA	TTT	GTT	TGT	CCA	ACA	GAA	ATG	ATT	GGA	TAT	334
79	Y	P	L	D	W	T	F	V	C	I	D	E	M	I	G	Y	94
335	AGT	GAA	CTT	GCA	GGA	CAA	TTG	AAA	GAA	ATC	AAT	T	E	G	ATT	GGA	382
95	S	E	L	A	G	Q	L	K	E	I	N	C	E	V	I	G	110
383	GTG	AGT	GTA	GAT	TCA	GTT	TAT	TGT	CAT	CAA	GCA	TGG	TGT	GAA	GCA	GAT	430
111	V	S	V	D	S	V	Y	C	H	Q	A	G	A	A	D		126
431	AAA	AGT	AAA	GGA	GGA	GTA	GGA	AAG	TTG	ACA	TTC	CCA	TTA	GTA	TCA	GAT	478
127	K	S	K	G	G	V	G	K	L	T	F	P	L	V	S	D	142
479	ATT	AAG	AGA	TGC	ATT	TCT	ATC	AAA	TAT	GCA	ATG	TAT	AAT	GGA	GAA	GCA	526
143	I	K	R	C	I	S	I	K	Y	A	M	L	N	V	E	A	158
527	GGA	ATT	GCA	AGA	AGA	GGA	TAT	GTC	ATC	ATT	GAT	GAT	AAA	GGA	AAA	GTA	574
159	G	I	A	R	R	G	Y	V	I	I	D	G	A	G	K	V	174
575	AGA	TAC	ATT	CAA	ATG	AAT	GAT	GAT	GGA	ATT	GGA	AGA	TCA	ACG	GAA	GAA	622
175	R	Y	I	Q	M	N	D	D	G	I	G	R	S	T	E	E	190
623	ACA	ATC	AGA	ATA	GTT	AAA	GCA	ATT	CAA	TTG	AGT	GAT	GAA	CAT	GGA	GCA	670
191	T	I	R	I	V	K	A	I	Q	F	S	D	E	H	G	A	206
671	GTT	TGT	CCA	CTC	AAT	TGG	AAA	CCA	GGC	AAA	GAC	ACC	ATT	GAA	CCA	ACA	718
207	V	C	P	L	N	W	K	P	G	K	D	T	A	A	T		222
719	CCA	GAT	GGA	ATT	AAG	AAA	TAT	TTA	ACA	GCA	CAT	TAA	AAC	AAA	CAA	GAT	766
223	P	D	G	I	K	K	Y	L	T	A	H	*					234
767	AAT	TTA	ATA	CAA	ATT	ATT	TTA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	804

FIG. 1. Nucleotide sequence of the p48 cDNA clone and deduced amino acid sequence of the coding region. An asterisk indicates the translation stop codon. The sequence is 804 nucleotides in length with 52 untranslated nucleotides at the 5' end and 50 untranslated nucleotides at the 3' end.

gen (100 $\mu\text{g}/\text{ml}$), or purified native or recombinant 29-kDa protein (0.1 to 10 $\mu\text{g}/\text{ml}$) was utilized in culture medium for a total volume of 0.2 ml per well. Ten replicate samples were studied. After 4 days of incubation at 37°C in 5% CO_2 , plates were pulsed with [^3H]thymidine ([methyl- ^3H]thymidine; Dupont, New England Nuclear Products, Boston, Mass.) at 1 μCi per well and harvested 24 h later with a cell harvester (Skatron Inc., Sterling, Va.) onto glass filter strips (Skatron Inc.). The filter disks were placed in disposable scintillation vials (Skatron Inc.), scintillation cocktail was added (Beckman Ready Value Scintillation Cocktail; Beckman Laboratories Inc., Fullerton, Calif.), and after vigorous shaking, ^3H activity was determined in a scintillation counter (Beckman LS7500). The results were expressed as blastogenic indices.

Immunization of gerbils with recombinant fusion protein and intrahepatic challenge with axenic trophozoites. Fifty- to 60-day-old Mongolian male gerbils were immunized intraperitoneally with 10 μg of the recombinant fusion protein emulsified in Titermax (50:50; Vaxcel, Norcross, Ga.); three immunizations were given, with a 2-week interval between injections. Control gerbils were sham immunized with Titermax or saline over three separate experiments. Gerbil sera were collected 35 days after immunization by puncture of the retro-orbital plexus of the eye with a capillary tube. The titer of serum antibody to the fusion protein was determined by ELISA.

Amebic liver abscesses were induced by direct inoculation of 5×10^5 axenic *E. histolytica* trophozoites into the liver by the method of Chadee and Meerovitch (2). Under metofane anesthesia, a laparotomy was performed under sterile conditions. Amebas were suspended in 0.1 ml of TYI culture medium and inoculated under the capsule of the liver by multiple injections with a 26-gauge needle. Gerbils were sacrificed 14 days postinoculation, and the formation of liver abscesses was determined. Abscesses were aspirated under sterile conditions for microscopy and axenic culture to document the presence of *E. histolytica* trophozoites and then dissected from the liver (the weights of abscesses and nonabscessed liver tissue were determined). Vaccine efficacy was calculated as $100 \times [1 - (\text{percent immunized animals with liver abscess/percent control animals with liver abscess})]$.

Statistical analysis. Significances of differences were determined by Student's *t* test or Fisher's exact test as appropriate.

RESULTS

Cloning and sequencing of cDNA encoding the 29-kDa antigen. The cDNA library was generated from *E. histolytica* HM1:IMSS poly(A)⁺ RNA. Of the 10^6 plaques screened with pooled human immune sera (sera from convalescent liver ab-

FIG. 2. Recognition of the purified recombinant fusion protein by human serum IgG antibodies as determined by ELISA. Optical density is indicated for the different patient groups, including normal Egyptian controls (Nor.Egypt.Con.), asymptomatic cyst passers (Asym.Cyst.Pass), amebic colitis patients, and amebic liver abscess (ALA) patients. Short horizontal lines represent the mean for each group, and the long horizontal line represents the cutoff point for positivity (mean + 2 standard deviations).

scs patients), 71 positive clones were isolated. Forty-four of the 71 positive clones were recognized by protein dot blot with the FP14 anti-29-kDa-protein monoclonal antibody. DNA hybridization with a 700-bp fragment of the gene encoding the 29-kDa antigen (22) determined that 51 of the 71 clones contained a cDNA insert encoding the amebic 29-kDa antigen. The sizes of the inserts were approximately 50 to 800 bp. The clone containing the longest insert, of approximately 800 bp, designated p48, was selected for further analysis. The full sequence of p48 was 804 nucleotides in length, including the entire coding region for the protein of 234 amino acids, 52 nucleotides at the 5' upstream region, and 50 nucleotides at the 3' downstream region (Fig. 1). The 714 bp from the 3' end of the p48 clone is completely homologous to the sequence

reported by Tachibana et al. (21). The sequence of the gene we cloned is homologous with the genomic DNA sequence up to the point 43 bp upstream from the translation initiation sites (21). The molecular mass deduced from the amino acid sequence of the gene for the 29-kDa protein is 26,000 Da.

Expression and purification of the 29-kDa protein in recombinant form. Expression of the protein in a pBluescript system provided insufficient material; therefore, we subcloned the gene for the 29-kDa protein from p48 into a pRSET expression vector driven by a T7 promoter. The fusion protein has a predicted molecular mass of 33 kDa. Two major contaminating proteins of 66 and 29 kDa were coeluted from the metal affinity chromatography column with the recombinant 33-kDa fusion protein. Therefore, to remove the contaminant, immunoaffin-

FIG. 3. Gerbil serum IgG antibody responses to the recombinant fusion antigen determined by ELISA. Gerbils received three inoculations with 10 μ g of recombinant fusion protein mixed with Titermax adjuvant (50:50), and studies were performed at a 1:500 dilution of serum. The horizontal bars represent the mean optical density for each group. Immunized gerbils (●) had significantly greater serum IgG antibody responses ($P < 0.01$) than the control group (○).

ity chromatography was performed with an anti-29-kDa-protein monoclonal antibody. Successful purification of the recombinant fusion protein following this two-step procedure was demonstrated by SDS-PAGE and immunoblotting (data not shown). The fusion protein migrates in monomeric and dimeric forms, as observed previously in studies of the 29-kDa antigen (7).

Antigenicity of the recombinant fusion protein. Antigenicity of the recombinant fusion protein was evaluated by ELISA with sera from U.S. controls, Egyptian controls, Egyptian patients with amebic colitis (113 cases), individuals residing in South Africa with amebic liver abscess (101 cases), and subjects with asymptomatic *E. dispar* (50 cases) or *E. histolytica* infection (4 cases). Over 80% of patients with amebic liver abscess had serum IgG antibodies to the recombinant fusion protein in comparison with uninfected U.S. or Egyptian controls (Fig. 2). In contrast, only a small number of patients with amebic colitis or asymptomatic *E. histolytica* infection possessed serum antibodies to the recombinant antigen. Serologic

specificity for the hepatic state of invasive amebiasis were also found with native 29-kDa protein, being positive in 78% of patients with amebic liver abscess compared with only 7% of patients with amebic colitis ($P < 0.01$).

Purified native 29-kDa antigen and the recombinant fusion protein induced proliferation of lymphocytes obtained from patients with amebic liver abscess in a dose-dependent manner ($P < 0.05$). Six control subjects and seven patients with acute or convalescent liver abscess were studied. Recombinant 29-kDa antigen resulted in blastogenic indices of 1.94 to 6.62 in patients compared with 1.43 to 2.57 in control subjects (at 1.0 and 10 μ g/ml, respectively) ($P < 0.05$). Native 29-kDa antigen induced a higher blastogenic response than the recombinant protein, i.e., 3.28 and 7.79 at 0.1 and 10 μ g/ml, respectively ($P < 0.01$ compared with control subjects).

Immunogenicity and vaccine efficacy of the recombinant fusion protein. Three intraperitoneal immunizations with recombinant protein (10 μ g) and Titermax as adjuvant elicited a serum IgG antibody responses in all gerbils studied (Fig. 3).

FIG. 4. Immunoblot of immune gerbil serum to total native parasite protein. SDS-PAGE and immunoblotting of immune gerbil serum demonstrated recognition of the 29-kDa protein and a 60-kDa dimer among total parasite protein (lane A). Immunoblotting with an anti-29-kDa-protein monoclonal antibody to total parasite protein as a positive control (lane B) revealed identification of the same protein bands as observed with immune gerbil serum. Control gerbil sera had no reactivity to any of the proteins studied (not shown).

The purity of the immunogen and the specificity of the gerbil antibody response were indicated by immunoblots of serum antibody to the recombinant fusion protein (data not shown) and total native parasite protein (Fig. 4). Gerbil immune sera and an anti-29-kDa-protein monoclonal antibody each recognized identically migrating 29- and 60-kDa native amebic proteins, with the 60-kDa band being a dimer of the 29-kDa protein.

To determine if this surface antigen was protective as a subunit vaccine, immunized gerbils were challenged intrahepatically with 5×10^5 virulent axenic HM1:IMSS trophozoites. We found that 30% of the gerbils immunized with the purified recombinant fusion protein developed amebic liver abscess upon challenge, compared with 59 and 65% of gerbils in the two control groups ($P < 0.05$) (Table 1), providing a vaccine efficacy of 54%. There was no significant difference between the sizes of the liver abscesses in any of the three groups (Table 1).

DISCUSSION

The 29-kDa protein of *E. histolytica* is a highly conserved antigen with epitopes specific to pathogenic *E. histolytica* isolates (13, 22). We demonstrated that a recombinant fusion protein translated from cDNA that encodes the entire 29-kDa protein has serologic specificity for the hepatic phase of disease, possesses conserved T-cell epitopes, and is immunogenic and partially protective as a subunit vaccine in the gerbil model of amebic liver abscess.

TABLE 1. Protection in the experimental gerbil model of amebic liver abscess (ALA) by immunization with the recombinant 29-kDa fusion protein

Treatment	No. of gerbils:		% Occurrence of ALA
	With ALA	Without ALA	
Saline	15	8	65
Adjuvant only (Titermax)	13	9	59
Recombinant 29-kDa antigen (10 µg in Titermax)	7	16	30 ^a

^a $P < 0.05$ compared with saline and Titermax controls.

The native 29-kDa *E. histolytica* antigen is extremely labile, possibly because of autoproteolysis (13). The level of expression of the 29-kDa protein in the T7 system was commensurate with that found with other *E. histolytica* proteins (17). Comparison of the codon adaptive index (analyzed by DNA Strider 1.1) of the gene for the 29-kDa protein with that of the DNA for a cysteine-rich section of the galactose-inhibitable lectin heavy subunit reveals low values for the 29-kDa protein gene. Despite the usual correlation between the abundance of a protein and its pattern of codon usage, poor expression of a cloned gene in *E. coli* may not be completely attributed to the infrequent use of rare codons (14). Decreased expression of protein could also be due to instability of the target mRNA, toxicity of recombinant products for bacteria, instability of the target protein, and a relatively high level of translational frameshifting and interfering structures in the mRNA (19).

We found that serum IgG responses to the recombinant fusion protein and the native 29-kDa antigen were present in most subjects with amebic liver abscess but in few with amebic colitis. Our results conflict somewhat with the work of Flores et al. (7), who reported the presence of serum antibodies to the 29-kDa protein in patients with amebic colitis. However, Flores et al. utilized a somewhat different ELISA methodology and studied 7 (compared with our 113) patients with amebic colitis and only 33 (compared with our 101) patients with amebic liver abscess. Since native and recombinant 29-kDa proteins gave similar results in our hands, we conclude that the serologic specificity for hepatic amebiasis is not unique to the nonglycosylated recombinant fusion protein. The dominant recognition of cDNA clones encoding the 29-kDa antigen by sera from liver abscess patients independently confirms the serologic findings. It is unclear what mechanism is responsible for this specificity; in contrast to *Leishmania* or *Malaria* species, there is no known change in the life cycle of *E. histolytica* within host tissues.

The cellular localization of the 29-kDa antigen is controversial. Tachibana et al. (21) localized the protein within the nucleus and in the cytoplasm by using immunofluorescence with formalin-fixed cells and binding by a monoclonal antibody (21). This finding is further supported by the results of Bruchhaus and Tannich (1). They analyzed the genomic sequence of the gene for the 29-kDa protein and suggested that the antigen is intracellular because of the lack of a hydrophobic leader sequence present at the N-terminus region. In addition, the nucleotide sequence revealed substantial homology to a class of polypeptides found in prokaryotic organisms that inactivate hydrogen peroxide (1). However, the evidence provided by Torian and coworkers (6, 13, 22) for surface localization is convincing. They demonstrated the peripheral membrane localization by iodination with immunoprecipitation, by immunofluorescence with live trophozoites, and by immunoelectron microscopy. Flores et al. (6) demonstrated that the 29-kDa protein forms a 60-kDa dimer through disulfide bonds and forms a high-molecular-mass oligomer(s) on the cell surface. The 29-kDa antigen is the major accessible free thiol-containing surface protein of *E. histolytica*. Possibly, the greater exposure in the liver to host cell-mediated immune responses or the existence of tolerance to the antigen in gut-associated lymphoid tissues is the mechanism by which the serum antibody response develops predominantly during hepatic and not intestinal amebiasis.

The 33-kDa recombinant fusion protein is highly immunogenic with Titermax as the adjuvant, inducing serum antibodies which exclusively recognized the native 29-kDa antigen. This was an important finding, indicating a greater feasibility for use as a vaccine immunogen. Immunization with the liver abscess-

specific recombinant antigen provided 54% vaccine efficacy in the gerbil model of amebic liver abscess. Although recombinant 29-kDa protein is not able to provide 100% protection in the gerbil model of amebic liver abscess, the 29-kDa protein may be best suited for use in a combination vaccine with other antigens. We recently demonstrated that a recombinant cysteine-rich section of the 170-kDa subunit of the 260-kDa galactose-inhibitable adherence protein is protective in the gerbil model of amebic liver abscess (17). Stanley and coworkers confirmed that finding (26) and found protection in the gerbil model with a recombinant serine-rich amebic antigen (25) and in the SCID mouse with polyclonal anti-*E. histolytica* antiserum (4). In the future, combinations of protective epitopes from multiple antigens may prove most effective against both hepatic and colonic disease.

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