

Identification of an Epitope on the *Entamoeba histolytica* 170-kD Lectin Conferring Antibody-mediated Protection against Invasive Amebiasis

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Summary

The emergence of multidrug-resistant organisms and the failure to eradicate infection by a number of important pathogens has led to increased efforts to develop vaccines to prevent infectious diseases. However, the nature of the immune response to vaccination with a given antigen can be complex and unpredictable. An example is the galactose- and *N*-acetylgalactosamine-inhibitable lectin, a surface antigen of *Entamoeba histolytica* that has been identified as a major candidate in a vaccine to prevent amebiasis. Vaccination with the lectin can induce protective immunity to amebic liver abscess in some animals, but others of the same species exhibit exacerbations of disease after vaccination. To better understand this phenomenon, we used recombinant proteins corresponding to four distinct domains of the molecule, and synthetic peptides to localize both protective and exacerbative epitopes of the heavy chain subunit of the lectin. We show that protective immunity after vaccination can be correlated with the development of an antibody response to a region of 25 amino acid residues of the lectin, and have confirmed the importance of the antibody response to this region by passive immunization studies. In addition, we show that exacerbation of disease can be linked to the development of antibodies that bind to an NH₂-terminal domain of the lectin. These findings are clinically relevant, as individuals who are colonized with *E. histolytica* but are resistant to invasive disease have a high prevalence of antibodies to the protective epitope(s), compared to individuals with a history of invasive amebiasis. These studies should enable us to develop an improved vaccine for amebiasis, and provide a model for the identification of protective and exacerbative epitopes of complex antigens.

The intestinal protozoan parasite *Entamoeba histolytica* is capable of invading and destroying human tissues, leading to potentially life-threatening diseases such as hemorrhagic colitis and extraintestinal abscesses. It is estimated that *E. histolytica* is responsible for about 50,000,000 cases of invasive amebiasis annually, resulting in 100,000 deaths, and thus rates among the leading parasitic causes of death, surpassed only by malaria and schistosomiasis (1). Morbidity and mortality associated with amebic infection have persisted despite the availability of effective therapy, suggesting that interventions designed to reduce or eliminate disease are needed. In principle, these objectives could be achieved by the introduction of a suitable vaccine. Since humans are the only relevant host for *E. histolytica*, an effective vaccination program could potentially eradicate amebiasis.

One of the leading candidates for a vaccine to prevent amebiasis is the galactose- and *N*-acetylgalactosamine-inhibitable lectin. The structure and function of this ameba surface receptor has been studied in considerable detail (for review see references 2 and 3). It is a membrane-associated glycoprotein with disulfide-linked subunits of a molecular mass of 170 and 35 kD, respectively (4). Both subunits of the receptor have been cloned, and their primary structures were deduced from cDNA and genomic sequences (5–8). The galactose- and *N*-acetylgalactosamine-inhibitable lectin appears to play a key role in amebic pathogenesis. It mediates adherence to colonic mucins (which may be important in intestinal colonization) and mediates binding to host cells (2, 9, 10). Adherence to host cells is critically important in the pathogenesis of intestinal disease and amebic liver abscess, since the killing of cells by amebae is contact dependent (3). Consistent with its role in mediating adherence to target cells, antibodies to certain epitopes on the galactose- and *N*-acetylgalactosamine lectin can inhibit

Dedicated to Dr. Hans J. Müller-Eberhard, Professor and Director, Institute of Molecular Medicine, The University of Texas-Houston, on the occasion of his seventieth birthday.

amebic adherence to target cells. However, it has also been found that antibodies to other epitopes of the lectin can enhance binding of ameba trophozoites to mammalian cells (11). In addition, epitopes of the lectin have been implicated in cell-mediated immune responses to amebae (12), and evidence exist that the ability of *E. histolytica* to resist complement lysis is mediated by a CD59-like domain of the ameba lectin (13).

The purified native galactose- and *N*-acetylgalactosamine-binding lectin has been used to vaccinate gerbils to protect them against amebic liver abscess (14). Although vaccination was protective in most animals, in others there was evidence for a significant increase in liver abscess size, suggesting that the immune response to the lectin could also exacerbate disease. Because of the vaccine potential of this molecule and its many putative functions, we were interested in identifying protective and exacerbative epitopes of the lectin, and in determining whether protective or exacerbative epitopes could be correlated with functional regions of the molecule. Here we have used four nonoverlapping recombinant proteins spanning the sequence of the extracellular region of the heavy chain subunit of the galactose- and *N*-acetylgalactosamine-inhibitable lectin to demonstrate that immunization with two of the domains can provide protection against invasive amebiasis, that vaccination with the third domain is completely ineffective, and that vaccination with the fourth domain actually exacerbates amebic liver abscess formation. Strikingly, these results can be shown to be dependent on the antibody response, since passive immunization of SCID mice with serum derived from animals vaccinated with the individual domains reproduces the results seen with active immunization. Using synthetic peptides we have also been able to demonstrate that a protective immune response after vaccination with one of the recombinant lectin domains is most likely based on the development of an antibody response to an epitope(s) contained within a stretch of 25 amino acid (aa)¹ residues. The clinical relevance of these data has been indicated by our findings that serum samples from asymptomatic individuals colonized with *E. histolytica* who appear to be resistant to invasive amebiasis show a high level of reactivity with one of the protective domains, while only a few individuals with a history of invasive amebiasis show antibodies to this domain. Finally, we have found that one of the protective domains of the molecule is also a potent T cell mitogen, suggesting that it contains the carbohydrate binding site of the ameba lectin.

Materials and Methods

Expression and Purification of Recombinant Proteins. The cDNA sequence of clone ZAP-170/4 previously isolated in our laboratory (5) was digested with the restriction endonucleases BglII or Sau3A. Respective fragments encoding aa sequences 1-436, 436-624, 799-939, and 939-1,053, respectively, were ligated into the

prokaryotic expression vector pJC20 (15) and transformed into *Escherichia coli* strain BL21 DE3 (plys S). Expression of recombinant proteins was achieved by induction with 0.3 mM isopropyl- β -D-thiogalactopyranoside. Subsequently, bacteria were sedimented and redissolved in sonication buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.3) in the presence of 0.3 mg/ml lysozyme. After one round of freezing and thawing, bacteria were ultrasonicated on ice at 30 W for 10 min. The suspension was centrifuged at 8,000 *g* for 20 min and the pellet was redissolved in sonication buffer supplemented with 0.1% Triton X-100 followed by two rounds of stirring at room temperature (RT) for 1 h and centrifugation at 8,000 *g*. The resulting pellet was dissolved in β -mercaptoethanol containing loading buffer, heated, and loaded onto a continuous preparative SDS-gel electrophoresis (Prep Cell, model 491; Bio Rad Labs, Hercules, CA) using a 13% gel matrix. Migrating proteins were eluted in 3-ml fractions. To remove SDS from SDS-protein complexes, pooled fractions containing the recombinant proteins only were dialyzed at 4°C overnight against a buffer containing 6 M guanidinium-HCl, 50 mM NaCl, and 50 mM Tris-HCl, pH 7.5. SDS, which formed an opaque precipitate, was removed by ultracentrifugation at 140,000 *g*. The supernatant was dialyzed extensively against 25 mM NaCl/50 mM Tris-HCl buffer, pH 7.5, with decreasing guanidine concentration until guanidine was completely removed. Identity of the purified recombinant proteins was determined by NH₂-terminal sequencing using a gas-phase protein sequencer (model 473A; Applied Biosystems, Foster City, CA). Purity of proteins was assessed by reversed phase HPLC.

ELISA for the Detection of Antilectin Antibodies in Human Sera. This procedure was performed essentially as previously described (16) using 130 ng of recombinant protein and human sera in a dilution of 1:400.

Cultivation of Cells. Trophozoites of the *E. histolytica* isolate HM-1:IMSS were grown axenically in TYI-S-33 medium (17). Virulence was maintained by gerbil liver passage once per month. For adherence assays, trophozoites in the logarithmic phase of growth were detached by chilling of ice, pelleted by centrifugation at 500 *g* for 5 min, washed twice with RPMI, and subsequently resuspended in RPMI containing 0.5% BSA and 25 mM Hepes, pH 7.5. Chinese hamster ovary (CHO) cells were grown in RPMI in the presence of 10% fetal calf serum, penicillin (100 U/ml), and streptomycin sulfate (100 μ g/ml). Cells were released by trypsinization (0.25% trypsin/EDTA; GIBCO BRL, Gaithersburg, MD).

Immunization of Rabbits and Gerbils. White New Zealand rabbits were immunized subcutaneously with 250 μ g of recombinant protein emulsified in complete Freund's adjuvants. Booster immunizations were performed every 14 d with the same amount of protein using incomplete Freund's adjuvants until a significant antibody titer of at least 1:800 against the respective proteins was obtained as determined by ELISA.

Adult female gerbils (*Meriones unguiculatus*) were immunized intraperitoneally with 50 μ g of recombinant protein emulsified with complete Freund's adjuvant followed by two booster immunizations after 14 and 28 d, respectively, using the same amount of protein emulsified in incomplete Freund's adjuvants.

Induction of Amebic Liver Abscess in SCID Mice and Gerbils. SCID mice were treated according to the method developed by Cieslak et al. (18). Each animal received 200 μ l of rabbit immune or preimmune serum intraperitoneally 24 h before challenge. Passively immunized SCID mice were challenged with 10⁶ and actively immunized gerbils with 10⁵ virulent *E. histolytica* trophozoites according to the method described by Chadee and Meerovitch

¹Abbreviations used in this paper: aa, amino acid; CHO, Chinese hamster ovary; RT, room temperature.

(19). The animals were fasted for 24 h and subsequently anesthetized by intramuscular application of a combination of ketaminhydrochloride and xylazine. Laparotomy was performed by a vertical incision of about 1 cm to visualize the liver. Amebae were injected in a volume of 100 μ l into the left liver lobe. Peritoneum and abdominal wall were closed by catgut sutures and the skin was closed using clips. 7 d later, animals were killed, and the liver was entirely removed, sectioned, and the sizes of abscesses or their weight relative to total liver weight was determined.

Solid-phase Enzyme Immunoassay for the Detection of Antibodies to Synthetic Peptides. Synthetic overlapping 170CR2-derived 25-mer peptides were prepared by Pacemaker (Affinity Research, Exete, U.K.). The following peptides were used: 170CR2-PEP1, NH₂-DPNFDCQPIECKIQEI-VITEKDGK; 170CR2-PEP2, NH₂-IVITEKDGKTTTTVKD-GTKTTCDTN; 170CR2-PEP3, NH₂-DGTKTTCDTNNKRIEDARKAFIEGK; 170CR2-PEP4, NH₂-DARKAFIEGKEGIEQVECASTVCQN; 170CR2-PEP5, NH₂-VECASTVCQN-DNSCPIIADVEKCNQ; 170CR2-PEP6, NH₂-IADVEKCNQNTVEDYDGCKAMTGE; 170CR2-PEP7, NH₂-YGCKAM-TGECDDTTYLCKFVQLTDD. Lyophilized peptides were solubilized in 25 mM ammonium sulfate, pH 4.5, diluted to a concentration of 10 μ g/ml in PBS, and 50 μ l/well were coated to maxisorb microtiter plates (Nunc, Roskilde, Denmark) at RT overnight. Plates were washed three times with PBS supplemented with 0.1% Tween 20. Blocking was performed with 100 μ l of PBS supplemented with 20% fetal calf serum at RT for 3 h. Subsequently, plates were washed and the gerbil sera diluted in PBS containing 10% fetal calf serum was added. After incubation at RT for 2 h, plates were washed and peroxidase-conjugated rabbit anti-hamster IgG (Dianova GmbH, Hamburg, Germany) in a dilution of 1:600 was added and incubated at RT for 1 h. Subsequently, plates were washed three times and the chromogenic substrate *o*-phenyldiamine was added. After incubation for 5 min, the color reactions were stopped with 2 M H₂SO₄ and measured using an automatic plate reader (MR 5000; Dynatech Labs. Inc., Chantilly, VA).

Adherence Assay. Amebic adherence to CHO cells was performed according to the method described by Ravdin and Guerant (9). In brief, ameba trophozoites were washed three times in RPMI and suspended to a final concentration of 10⁶ ml RPMI medium containing 0.5% BSA, 2 μ M calcein A (Calbiochem Corp., La Jolla, CA) and 25 mM HEPES, pH 7.5, and incubated at 37°C for 15 min. After one washing, trophozoites were incubated with the respective preimmune or immune rabbit sera on ice for 2 h. After two washings, 10⁴ trophozoites were centrifuged together with 10⁶ CHO cells at 150 *g* and incubated on ice for 2 h in a volume of 1 ml of RPMI buffer. Subsequently, rosette formation was visualized by microscopy. Rosette formation was defined as the percentage of ameba with at least three adherent CHO cells. All experiments were done in duplicate and performed four times.

Spleen Cell Proliferation Assay. Gerbil spleen cells were prepared and cultured in RPMI medium containing 1% l-glutamine, 10% fetal calf serum, and 50 μ g/ml gentamicin. Gerbils were killed, and the entire spleen was removed and transferred into 10 ml of medium. Subsequently, the spleen was disrupted and transferred to a tube. After 5 min, to allow pelleting of large particles, the supernatant was removed, transferred to another tube, and cells were pelleted by centrifugation with 150 *g* at 4°C for 5 min. After washing, cells were counted and transferred to 96 round-bottom microtiter plates at a density of 2 \times 10⁵/well. Plates were incubated in a humidified atmosphere with 5% CO₂ at 37°C. Recombinant proteins or respective controls were added and incubated

for 2 to 6 d. Subsequently, cells were pulsed with 0.4 mCi/well [³H]thymidine (Amersham Corp., Arlington Heights, IL) and incubated for an additional 12 h before harvesting on a filter using an automatic cell harvester. Thymidine incorporation was determined using an automatic scintillation counter. All assays were done in triplicates and performed three times. Culture medium, Con A and *E. histolytica* membrane extract served as controls. The latter was prepared by solubilizing of trophozoites in 0.8% octylglycopuranside and subsequent dialyzing of the lysate against PBS containing 0.05% octylglycopuranside.

Results

Reactivity of Human Sera to Recombinantly Expressed Polypeptides Derived from the *E. histolytica* 170-kD Lectin. Based on the main structural motifs of the *E. histolytica* 170-kD lectin, a full-length cDNA previously isolated in our laboratory was dissected into four nonoverlapping fragments. The derived aa sequences of these fragments, designated r170CP, r170PR, r170CR1, and r170CR2, respectively, represent the NH₂-terminal cysteine-poor region (aa residues 1–436), the pseudorepetitive part within the cysteine-rich region (aa residues 436–624), as well as two additional sections of the cysteine-rich region located within the COOH-terminal part of the molecule (aa residues 799–939 and 939–1,053, respectively) (Fig. 1). The four cDNA fragments were ligated into a prokaryotic expression vector which allowed the production of high amounts of the respective polypeptides as nonfusion proteins in *E. coli*. The recombinant lectin fragments were purified, and samples of purity >95%, as determined by HPLC, were used for further experiments (Fig. 1). Identity of each of the recombinant polypeptides was confirmed by protein sequencing.

Using ELISA, a total of 109 well-defined serum samples at a dilution of 1:400 were analyzed for their reactivity to

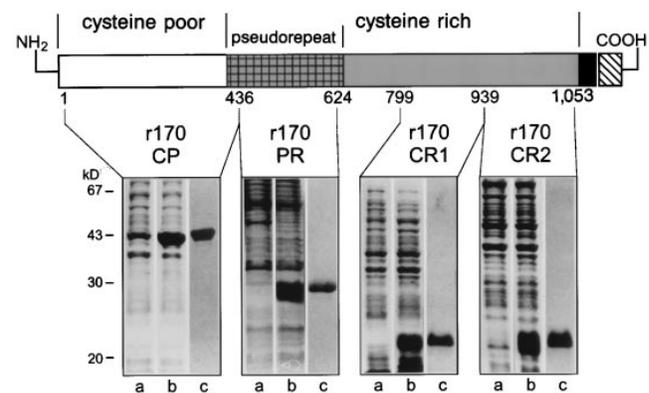


Figure 1. Expression and purification of recombinant fragments of the 170-kD subunit of the *E. histolytica* surface lectin. Shown are the structural domains of the protein as have been defined previously (12). Numbering refers to aa residues and indicate the boundaries of respective recombinant fragments. Below, expression and purification of each polypeptide is monitored by SDS-PAGE. Lanes: a, lysate of *E. coli* transformed with pJC20 (Coomassie blue stained); b, lysate of *E. coli* transformed with pJC20 in which the respective lectin cDNA fragments were subcloned (Coomassie blue stained); c, purified recombinant proteins (silver stained). Molecular size markers are indicated on the left.

Table 1. Recognition of Different Recombinant Fragments of the *E. histolytica* Galactose-inhibitable Surface Lectin by Human Sera

	Apparently no <i>E. histolytica</i> infection	Symptomatic <i>E. histolytica</i> infection	Asymptomatic <i>E. histolytica</i> infection	
r170CP	1/52 (2%)	48/48 (100%)	8/9 (89%)	(<i>P</i> = 0.16)*
r170PR	12/52 (23%)	27/48 (56%)	4/9 (44%)	(<i>P</i> = 0.39)
r170CR1	0/52 (0%)	37/48 (77%)	6/9 (67%)	(<i>P</i> = 0.39)
r170CR2	3/52 (6%)	5/48 (10%)	7/9 (78%)	(<i>P</i> <0.001)

**P* values between sera of patients with symptomatic and asymptomatic *E. histolytica* infection as determined by Fisher's exact test.

each of the four polypeptides. Serum samples were obtained from patients with amebic disease (*n* = 48) or from asymptomatic individuals excreting *E. histolytica* (*n* = 9). As a control, sera from healthy, apparently uninfected individuals (*n* = 20) as well as from patients with miscellaneous infectious diseases unrelated to amebiasis (*n* = 32) were included. Sera were collected from European individuals as well as from those living in areas endemic for amebiasis. The outcome of the assays is shown in Table 1. Depending on the antigen used, the results obtained differed substantially. All 48 serum samples (100%) collected from patients with amebic disease reacted with the NH₂-terminal cysteine-poor region (r170CP), whereas a reduced number did so with the other three recombinant antigens (56% with r170PR, 77% with r170CR1, and 10% with r170CR2). When compared with the 52 control sera, specificity for the detection of antiamebic antibodies was high and exceeded 94% with three of the four polypeptides. On the other hand, specificity was only 77% using r170PR as the antigen, suggesting that the pseudorepetitive region shares some common epitopes which are recognized by a substantial number of human sera. This result may explain previous findings, indicating that ~25% of sera from individuals apparently not infected with *E. histolytica* recognize the purified, natural lectin molecule (20). Besides the differences between the four recombinant antigens concerning sensitivity and specificity for the detection of antiamebic antibodies, a more striking finding became evident by compar-

ing the reactivities between sera collected from patients with amebic disease and those from asymptomatic *E. histolytica* carriers. Sera of both groups reacted to a similar extent with r170CP, r170PR, and r170CR1, but a marked difference was found for r170CR2. This polypeptide was recognized by 78% of sera from asymptomatic carriers, but only by 10% of sera from amebiasis patients (*P* <0.001).

Immunogenicity and Vaccine Efficacy of Recombinant Proteins in Gerbils. Adult female gerbils were immunized intraperitoneally with three doses of 50 µg each of the respective recombinant antigen, emulsified in complete or incomplete Freund's adjuvants. As a control, animals were immunized using Freund's adjuvants only. A total of three trials were performed, each comprising four to seven animals. Subsequently, specific antibodies were determined indicating that none of the controls, but all of the animals immunized with recombinant protein, developed a significant serum IgG antibody response to the respective antigen. Challenge of these animals by liver inoculation with 10⁵ virulent *E. histolytica* trophozoites showed clear differences between the groups. As shown in Table 2, all of the 17 sham-immunized control animals developed liver abscesses, whereas immunization with either r170PR or r170CR2 revealed total protection against liver abscess formation in 6 out of 16 (37.5%) and 10 out of 16 (62.5%) gerbils, respectively, as well as significant reductions in the size of abscesses in the remaining animals. Immunization of gerbils using the NH₂-terminal cysteine-poor region of the lectin (r170CP) gave

Table 2. Protection of Gerbils from Amebic Liver Abscess by Vaccination with Recombinantly Expressed Fragments of the 170-kD Lectin

Vaccine group	No. of gerbils with liver abscess/ No. of gerbils challenged	Percent protected	Size of liver abscesses <i>mm mean ± SD</i>
Buffer	17/17 (5/5, 5/5, 7/7)	0.0	6 ± 2
r170CP	15/16 (3/4, 5/5, 6/6)	6.7	9 ± 3*
r170PR	10/16 (3/5, 3/4, 4/7)	37.5 (<i>P</i> <0.05)	3 ± 2*
r170CR1	8/9 (4/5, 4/4)	11.1	5 ± 3
r170CR2	6/16 (2/5, 1/4, 3/7)	62.5 (<i>P</i> <0.001)	3 ± 1*

*Significant difference from buffer control (*P* <0.05) as determined by Fisher's exact test.

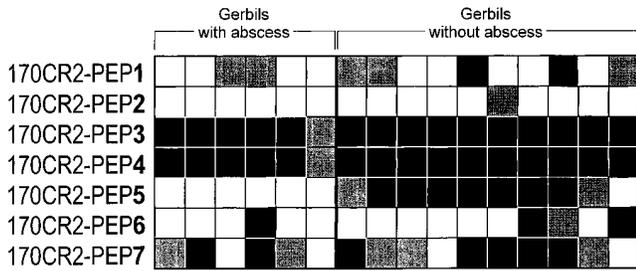


Figure 2. Seroreactivity of r170CR2-immunized gerbils to 170CR2-derived synthetic peptides. 16 gerbils were immunized with r170CR2 before challenge with virulent *E. histolytica* trophozoites. 6 of them developed abscesses, whereas 10 of them were found to be protected. Shown are antibody titers of the 16 gerbil immune sera to each of the seven overlapping 25-mer peptides (170CR2-pep1–7) spanning the entire 115 aa residues of 170CR2. *Open boxes*, sera that did not react with respective peptides at serum dilutions below 1:200; *gray boxes*, sera reactive at dilutions between 1:200 and 1:800; and *filled boxes*, sera reactive at dilutions above 1:800.

no protection and resulted in a significant increase in size of abscesses. Immunization with r170CR1 revealed neither protection nor a change in size of abscesses compared to controls. Titration of each of the various gerbil antisera against the respective antigen revealed no correlation between titer of antibodies and degree of protection.

Seroreactivity of r170CR2-immunized Gerbils to 170CR2-derived Synthetic Peptides. To determine whether the high degree of vaccine efficacy obtained by immunization of gerbils with r170CR2 could be mapped to a specific epitope, a set of seven overlapping peptides was synthesized spanning the entire 115 aa of the 170CR2 region. Each peptide consisted of 25 aa. Reactivity to each of the seven synthetic peptides was determined for each of the 16 sera collected

from the gerbils that had been immunized with r170CR2 (Fig. 2). Irrespective of whether sera were taken from gerbils that had developed abscesses, or from those that were protected, the two groups reacted to a similar extent with six of the seven peptides. High antibody titers were found against peptide 3 and 4, but none of the sera reacted with peptide 2. In contrast, a highly significant difference between the two groups of animals was found in response to peptide 5 ($P < 0.001$). None of the sera obtained from the six gerbils that had developed abscesses reacted with this peptide, even at the lowest dilution tested (1:100). In contrast, 9 out of the 10 animals that did not develop liver abscesses showed significant antibody titers against peptide 5. Two of the nine serum samples reacted at dilutions up to 1:200 and 1:400, respectively, and the remaining seven reacted at dilutions of at least 1:800 and some of them even at dilutions of up to 1:12,800.

Passive Transfer Studies in SCID Mice. To further validate the role of antibodies directed to the various regions of the 170-kD lectin in exacerbation or protection of disease, we performed passive immunization studies in SCID mice. Polyclonal rabbit antisera against the recombinant polypeptides were transferred into SCID mice 24 h before intrahepatic challenge with 10^6 virulent *E. histolytica* trophozoites. The antisera were generated by immunization of rabbits with r170CP, r170PR, and r170CR2, respectively. Booster immunizations were performed until antisera reacted to the respective antigen in a dilution of at least 1:1,600. Titration of the antiserum obtained from the r170CR2-immunized rabbit against the different synthetic peptides revealed high antibody titers against peptides 1, 3, 4, 6, and 7, respectively, but apparently no antibodies against peptide 2 and only a rather low antibody titer against peptide 5, which did not exceed 1:200 even after several booster immuniza-

Table 3. Protection of SCID Mice from Amebic Liver Abscess by Transfer of Rabbit Antisera Raised against Recombinantly Expressed Fragments of the 170-kD Lectin

Vaccine group	No. of SCID mice with liver abscess/ No. of SCID mice challenged	Percent protected	Percent liver abscessed in nonprotected mice
			<i>mean ± SD</i>
Preimmune	19/19 (4/4, 5/5, 5/5, 5/5)	0.0	17.1 ± 7.8
Anti-r170CP	8/8 (5/5, 3/3)	0.0	28.2 ± 12.6*
Anti-r170PR	5/8 (3/5, 2/3)	37.5	3.2 ± 1.2*
Anti-r170CR2a	8/8 (5/5, 3/3)	0.0	7.6 ± 3.8*
Anti-r170CR2a [‡]	2/5 (2/5)	60.0	5.5
Anti-r170CR2b	2/5 (2/5)	60.0	9.3
Anti-r170CR2c	3/5 (3/5)	40.0	8.3
Anti-r170CR2a [‡] + b + c	7/15 (2/5, 2/5, 3/5)	53.3	7.7 ± 1.8*

*Significant difference from preimmune control ($P < 0.05$), as determined by Fisher's exact test.

[‡]5× concentrated anti-r170CR2a antiserum with low antibody titer to peptide 5.

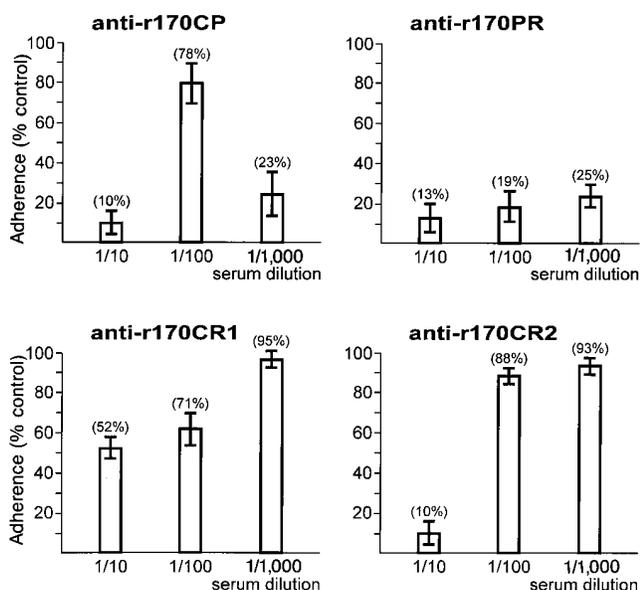


Figure 3. Inhibition of amebic adherence to CHO cells by antisera to recombinant antigens. *E. histolytica* trophozoites were treated with rabbit sera raised against recombinant polypeptides before incubation with CHO cells. Subsequently, the number of amebae with at least three adherent CHO cells (rosettes) was determined. Results were compared with those obtained with respective preimmune controls. Bars represent percentage of rosettes relative to respective controls at serum dilutions as indicated. Error bars represent standard deviations of four experiments performed in duplicate.

tions. Therefore, two additional rabbits were immunized with r170CR2. Fortunately, both developed substantial antibody titers to peptide 5 reacting at dilutions of up to 1:800 and 1:3,200, respectively. The various rabbit antisera, as well as respective preimmune control sera, were used for passive immunization in SCID mice. In addition, a 5× concentrated sample of the anti-r170CR2 antiserum which exhibited a low antibody titer to peptide 5 was included. As shown in Table 3, all of the 19 control animals, as well as all 8 mice which received anti-r170CP antiserum, developed amebic abscesses. The sizes of abscesses in the anti-r170CP group were significantly larger ($P < 0.05$) than those seen in the control animals, indicating that antibodies to the cysteine-poor region are exacerbating disease. In contrast, anti-r170PR and anti-r170CR2 antisera conferred total protection against liver abscess formation in 37.5 and 53.3% of mice, respectively. In addition, the sizes of abscesses in the remaining mice of these two groups were significantly smaller compared to controls. Interestingly, all eight SCID mice which received nonconcentrated anti-r170CR2 antiserum containing low amounts of antibodies to peptide 5 developed abscesses. However these abscesses were significantly smaller than those of controls (Table 3).

Inhibition of *E. histolytica* Adherence by Antisera. Rabbit antisera raised against the four recombinant polypeptides, as well as respective preimmune control sera, were investigated for their ability to inhibit adherence of *E. histolytica* trophozoites to CHO cells using a standard in vitro rosetting assay (Fig. 3). The results obtained indicated that com-

pared to controls, all of the immune sera were able to substantially inhibit adherence at low serum dilutions of 1:10. However, at higher dilutions of 1:100 or 1:1,000, this inhibitory effect was no longer detectable with anti-r170CR1 or anti-r170CR2. In contrast, anti-r170PR at dilutions of 1:100 or 1:1,000 strongly inhibited adherence by 81 and 75%, respectively. A rather unusual effect was obtained using the antiserum raised against the NH₂-terminal part of the lectin (anti-r170CP). Inhibition of adherence was only 22% at a serum dilution of 1:100, but increased to 77% at a dilution of 1:1,000.

Induction of Spleen Cell Proliferation. Previous studies have indicated that amebic cell extracts are able to stimulate cell proliferation (21), and that the galactose-inhibitable lectin is responsible for this activity (22). Therefore, we investigated cultures of spleen cells isolated from naive gerbils for their ability to proliferate in response to incubation with the recombinant polypeptides. As shown in Fig. 4, cells proliferated in the presence 30 μg/ml of *E. histolytica* membrane extracts, as well as in the presence of 2.5 μg/ml of r170PR, whereas no stimulation was achieved using the other three recombinant polypeptides. The main proliferative response was obtained within the first 48 h of incubation, and was comparable to the stimulation seen with the plant lectin concavalin A. Nonspecific stimulation which might be due to contamination by LPS was excluded since all preparations were found to contain less than 52 pg/ml of LPS, which is below the amount necessary to induce lymphocyte proliferation. In addition, specificity of proliferation by r170PR was further supported by the fact that treatment of the polypeptide with proteinase K, and subsequently with PMSF, completely inhibited stimulation, whereas control cells incubated with proteinase K and PMSF were not altered and could be stimulated by concavalin A.

Discussion

We made use of recombinantly expressed fragments of the *E. histolytica* 170-kD lectin to identify and characterize

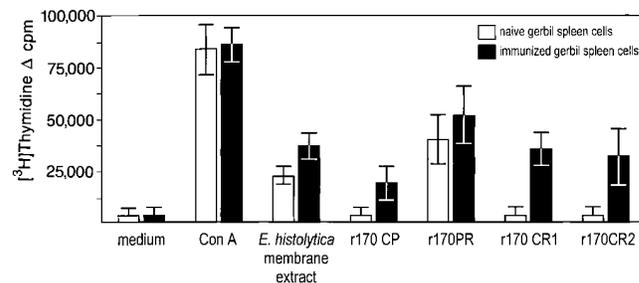


Figure 4. Proliferation of spleen cells isolated from naive (open bars) or immunized gerbils (closed bars) in response to recombinant antigens. Respective spleen cells were incubated in the presence of 2.5 μg/ml of the various recombinant polypeptides and blastogenesis was determined by [³H]thymidine uptake after 3 d (naive spleen cells) or 5 d (primed spleen cells) of incubation. The results are expressed as means of three experiments performed in triplicate with spleen cells of at least five animals. Culture medium, Con A, and *E. histolytica* membrane extract served as controls. The latter was used in a concentration of 30 μg/ml.

regions that might be suitable for use as a subunit vaccine against invasive amebiasis. A total of four separate fragments were investigated spanning almost the entire extracellular portion of the molecule. In contrast to previous publications using recombinant *E. histolytica* lectin, the polypeptides we used were expressed as nonfusion proteins, highly purified, and renatured by extensive dialysis against buffer, thus eliminating all of the detergent necessary to initially solubilize the recombinant proteins from *E. coli* lysates. Therefore, results obtained in this study may not necessarily complement those reported previously.

Depending on the polypeptide used, immunization of gerbils and subsequent liver inoculation of live *E. histolytica* trophozoites resulted either in protection against liver abscess formation or in development of enlarged abscesses. These effects were found to be mediated by specific antibodies, as evidenced by passive transfer experiments with respective antisera using the SCID mouse model. Previous vaccination trials in gerbils with purified, native *E. histolytica* lectin had revealed two different groups of responders. After challenge with *E. histolytica* trophozoites, 67% of immunized animals were found to be protected, whereas the remaining 33% developed larger abscesses compared to sham-immunized controls (14). Our results indicate that antibodies directed against the NH₂-terminal, cysteine-poor region of the lectin (r170CP) are responsible for the formation of larger abscesses, which might be due to antibodies reacting with adherence-enhancing epitopes. The presence of such epitopes on the ameba lectin has already been demonstrated using monoclonal antibodies (11, 23). In addition, it was reported that 36% of sera from patients with invasive amebiasis which had developed high antibody titers against the native *E. histolytica* lectin were found to increase adherence of the amebae to CHO cells (11). However, our in vitro studies on adherence of *E. histolytica* trophozoites to CHO cells did not reveal enhanced adherence in response to anti-r170CP antiserum. This antiserum strongly inhibited adherence at a dilution of 1:1,000 but interestingly, inhibition was drastically reduced at a dilution of 1:100. Therefore, we speculate that the anti-r170CP antiserum used in this study contains both adherence-enhancing, as well as adherence-inhibiting antibodies, which may compete for binding to the lectin, or which are present in different quantities, or bind with different affinity. Further dissections of r170CP will help to identify the epitope(s) responsible for exacerbation of amebic disease. Nevertheless, induction of larger abscesses by immunization with r170CP excludes this part of the lectin for use in a subunit vaccine.

In contrast to the NH₂-terminal, cysteine-poor part of the lectin, immunization of gerbils with the pseudorepetitive fragment of the cysteine-rich region (r170PR) or transfer of respective antibodies into SCID mice mediated some degree of protection against liver abscess formation. Most of the animals (62.5%) developed significantly smaller abscesses compared to respective controls, and the remaining showed no abscesses at all. Most notably, none of them developed larger abscesses. Our in vitro results indicate that r170PR has cell-binding activity. In contrast to the other

recombinant fragments, r170PR was found to specifically induce proliferation of nonprimed (naive) gerbil spleen cells, an effect comparable with that of the plant lectin Con A. Therefore, the pseudorepetitive part of the cysteine-rich region is likely to contain the sugar-binding domain, which is in line with the result that anti-r170PR antiserum strongly inhibited adherence of amebae to CHO cells. A number of monoclonal antibodies reacting with the ameba lectin have been reported (11, 23). All of them map to the cysteine-rich region. Three of them, which map to different fragments, were found to inhibit ameba adherence to CHO cells. According to the primary sequence, these fragments are separated by some hundred aa residues. Whether the three monoclonal antibodies map to different binding domains that may be located on the lectin or whether they induce a conformational change of the molecule has not been established. Nevertheless, one of the adherence inhibiting monoclonal antibodies maps to a fragment that overlaps with 170PR (23). Although a lectin fragment that contains the sugar-binding domain would appear to be an ideal candidate for an amebiasis vaccine, the use of r170PR might be disadvantageous because of its ability to induce spleen cell proliferation.

Highest vaccine efficacy was obtained by immunization with r170CR2, the relatively COOH-terminal-located, cysteine-rich fragment (amino acids [aa] 939–1053). From the 16 gerbils immunized with r170CR2, 62.5% were completely protected against amebic liver abscess formation, the size of abscesses in the remaining gerbils was significantly smaller, and none had larger abscesses compared to controls. In contrast, the results obtained by immunization with r170CR1 (aa 799–939) were identical to those of controls. Therefore, the cysteine-rich part of the lectin conferring protection can now be restricted to a region of 115 aa residues covered by 170CR2. The degree of protection by immunization with r170CR2 was comparable with those obtained in previous studies. Vaccinations of gerbils using a glutathione S transferase fusion protein containing most of the cysteine-rich, nonrepetitive region of the lectin (aa 649–1202) had revealed 81% vaccine efficacy (24), and LC3, a 52-kD histidine-tailed fragment of the cysteine-rich region (aa 785–1134) was found to protect 71% of immunized gerbils (25). In these two studies, a reduction in the sizes of abscesses in the nonprotected animals was not reported, although gerbils were killed and analyzed for liver abscess formation 14 d after challenge. In our study, vaccine efficacy was determined at day 7 after challenge. Since untreated animals are able to reverse some degree of pathology (19, 26), it might be possible that small abscesses would be cleared within the following 7 d and thus, the number of gerbils without abscesses may have increased in our study.

As in the aforementioned studies, we found no correlation between the degree of protection and the titer of antibodies using the recombinant polypeptides as antigen. However, further analysis of the antibody response against r170CR2 using 25-mer peptides as well as our passive transfer experiments in SCID mice revealed a strong corre-

lation between degree of protection and titer of antibodies to the sequence NH₂-VECASTVCQNDNSCPIADVE-KCNQ representing aa residues 999–1,023 of the 170-kD lectin.

Questions remain open about the mechanism by which antibodies to 170CR2-derived epitopes confer protection. Our results suggest that 170CR2 is not involved in adherence, thus an adherence-inhibiting effect can be excluded. Therefore, other functions that might be located on the lectin may be altered by anti-r170CR2 antibodies. Besides adherence, at least two additional functions have been considered to be present on the ameba lectin. Using monoclonal antibodies, fragments of the lectin have been identified that are involved in mediating (a) resistance of amebae to human complement (13), and (b) stimulation of tumor necrosis factor- α production by macrophages (12). In addition, one monoclonal antibody which did not inhibit adherence was found to decrease ameba-induced cell killing (23). Although the various antibodies recognized different epitopes, all of them map to fragments that overlap with 170CR2. Since all of the functions are strongly associated with the survival of *E. histolytica* trophozoites within the tissues, antibodies to 170CR2 may result in an accelerated clearance of the ameba from the tissues. Therefore, r170CR2 might constitute a vaccine which most likely will prevent amebic disease rather than preventing amebic colonization of the intestine. This assumption would be consistent with

our finding that in contrast to patients with amebic disease, most of the asymptomatic *E. histolytica* carriers have significant antibody titers to r170CR2. Unfortunately, only nine samples from asymptomatic carriers could be included in our study, since those individuals are extremely rare. Nevertheless, even with the small number of samples, the results obtained were highly significant ($P < 0.001$), suggesting that antibodies to r170CR2 are able to confer protection against invasive amebiasis, not only in artificially infected rodents, but also in naturally infected humans. Further analyses of r170CR2 and, in particular, immunization studies in monkeys will prove whether this polypeptide constitutes a suitable subunit vaccine to prevent invasive amebiasis in primates.

The work we have outlined here describes a successful approach to identifying both protective and exacerbative epitopes from the galactose- and *N*-acetylgalactosamine-inhibitable lectin, a major candidate in a vaccine for amebiasis. As efforts to develop vaccines for a variety of infectious and noninfectious diseases continues, we anticipate that other antigens will be found that induce complex immune responses, resulting either in protective immunity or exacerbating disease. A key element in understanding these responses and designing better vaccines will be the ability to identify, at the peptide level, the location of protective and exacerbative epitopes.

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