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Protection of Gerbils from Amebic Liver Abscess by Immunization with a Recombinant Protein Derived from the 170-Kilodalton Surface Adhesin of *Entamoeba histolytica*

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The protozoan parasite *Entamoeba histolytica* causes extensive morbidity and mortality worldwide through intestinal infection and amebic liver abscess. Here we show that vaccination of gerbils, a standard model for amebic liver abscess, with recombinant proteins derived from the 170-kDa galactose-binding adhesin of *E. histolytica* and the serine-rich *E. histolytica* protein or a combination of the two recombinant antigens provides excellent protection against subsequent hepatic challenge with virulent *E. histolytica* trophozoites.

The intestinal protozoan parasite *Entamoeba histolytica* infects more than 500,000,000 people and causes approximately 50,000 deaths yearly. Many of those deaths result from the major extraintestinal complication of amebiasis, amebic liver abscess. Prevention of amebiasis through vaccination may be a feasible goal. Animals immunized with crude preparations of *E. histolytica* lysates are protected against the development of amebic liver abscess after direct hepatic inoculation of amebic trophozoites (4, 5, 7, 9, 16). In addition, while the evidence is limited, epidemiologic studies suggest that patients who are cured of amebic liver abscess may be less likely to develop a subsequent infection than members of the general population (3, 15). One possible limitation of vaccine studies involving *E. histolytica* is the inability to obtain the quantities of amebic proteins necessary for immunization.

The 170-kDa galactose-specific adhesin is one of the major surface antigens of *E. histolytica* (13). The 170-kDa molecule has been implicated in the binding of amebae to mammalian cells through galactose-binding activity (11) and has been proposed to play a role in the resistance of amebae to complement (1). Petri and Ravdin have shown that immunization of gerbils with the native 170-kDa molecule provides protection against amebic liver abscess (10). We recently reported that gerbils immunized with a recombinant version of a different surface antigen of amebae, the serine-rich *E. histolytica* protein (SREHP), are protected against amebic liver abscess after direct hepatic inoculation of virulent trophozoites (18). In two trials, recombinant SREHP present as a maltose-binding protein (MBP) fusion protein (SREHP/MBP) protected 64 and 100% of vaccinated gerbils; no protection was found in MBP- and phosphate-buffered saline (PBS)-immunized control animals. We have now used this approach to examine the vaccine potential of a recombinant version of the 170-kDa galactose-specific adhesin. We immunized gerbils with a recombinant protein containing the cysteine-rich (CR) domain of the 170-kDa molecule as a glutathione-*S*-transferase (GST) fusion protein (170CR/GST) (19), the recombinant SREHP/MBP preparation, or a combination of the two fusion proteins (170/SR) and examined the resistance of immunized and control animals to direct hepatic inoculation with virulent amebae. Here we report that immunization with the 170CR/GST protein can protect gerbils from amebic liver abscess and that the protective efficacy of vaccination with 170CR/GST is similar to that seen with SREHP/MBP or 170/SR.

The expression of the 170-kDa surface adhesin and its three major domains as pGEX-encoded (GST) fusion proteins was described elsewhere (19). (The sequence used corresponds to the product of the *hgl-3* gene, based on the terminology of Purdy et al. [12]). In this study, we used the pGEX-170CR construct, which encodes amino acids 649 to 1202 of the 170-kDa adhesin fused to GST (19). This domain of the 170-kDa adhesin includes the region implicated in amebic complement resistance (1) and is highly immunogenic (the 170CR/GST fusion protein was recognized by the serum of 90% of patients with amebic liver abscess) (19). The 170CR/GST fusion protein was purified by lysing isopropyl-β-D-thiogalactopyranoside (IPTG)-induced pGEX-170CR-expressing *Escherichia coli* in 1% Triton X-100–PBS–10 mM EDTA–0.1 mM dithiothreitol–0.1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.)–0.1 mM benzamidine–0.4 mM leupeptin. After two 30-s sonication bursts, the lysate was centrifuged at 9,000 rpm for 20 min at 4°C. The resulting pellet was dissolved in 8 M urea–0.1 M glycine (pH 9.0); after centrifugation at 9,000 rpm (9,700 × *g*) for 20 min, the supernatant was loaded on Sephadex G-25-150, and the column was eluted with PBS. A fraction containing the 170CR/GST protein was identified by Coomassie blue staining and Western blotting (immunoblotting) of fractions separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The recombinant GST protein was expressed with pGEX-KG as previously described (19) and purified on glutathione-agarose as previously described (6). The expression and purification of the recombinant SREHP/MBP fusion protein (which contains amino acids 10 to 222 of the SREHP mole-

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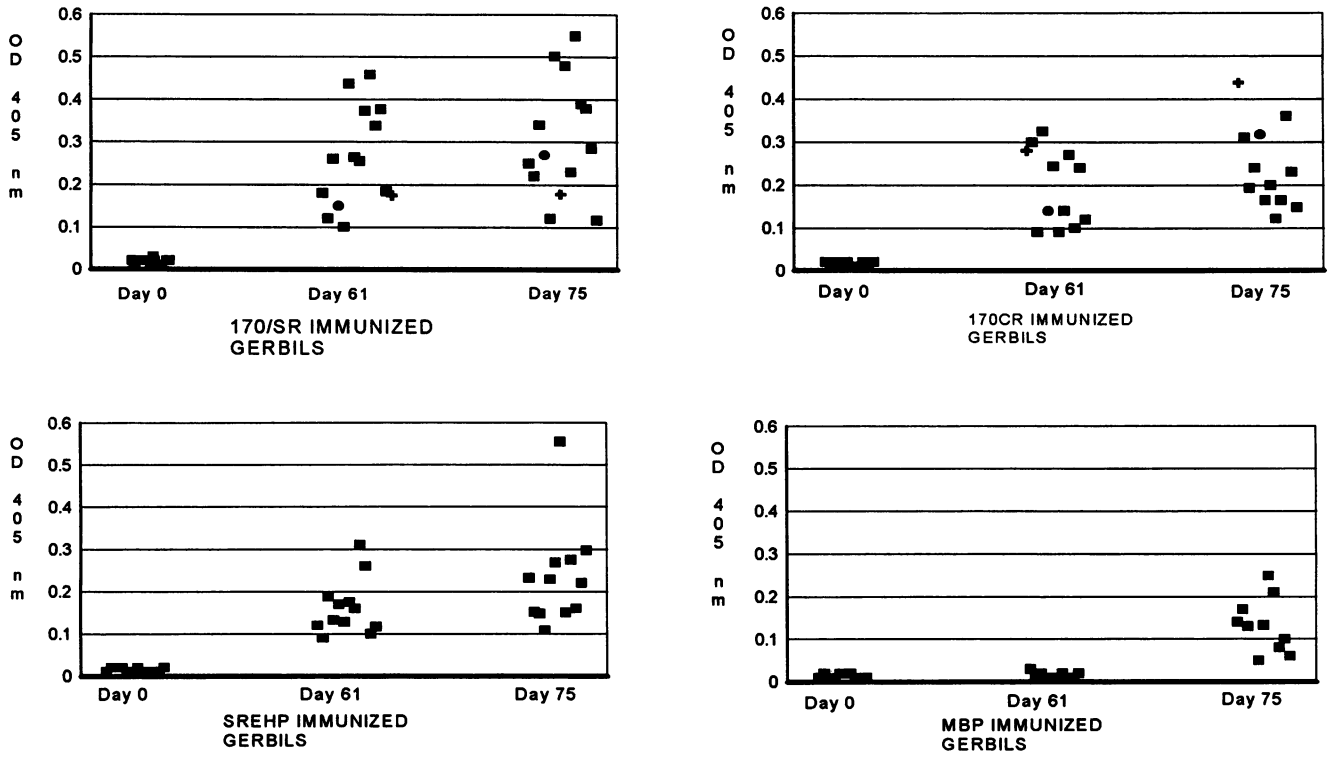

FIG. 1. Recognition of amebic lysates by serum (diluted 1:1,000) from 170CR/GST-, SREHP/MBP-, 170/SR-, and MBP-vaccinated gerbils at day 0, day 61 (before challenge with amebae), and day 75 (time of sacrifice), as measured by an ELISA. The values for the two gerbils that developed amebic liver abscesses in the 170CR/GST-vaccinated group and the 170/SR-vaccinated group are shown as + and ●. OD, optical density.

cule) and the MBP molecule with the pMAL system (New England Biolabs, Beverly, Mass.) have been described in detail elsewhere (8, 18).

We immunized adult female gerbils aged 6 to 8 weeks (Harlan Sprague Dawley, Indianapolis, Ind.) with 150 μg of fusion protein (170CR/GST [n = 12], SREHP/MBP [n = 12], and 170/SR [n = 14]), GST (n = 10), or MBP (n = 10) in a volume of 75 μl and an equal volume of complete Freund's adjuvant intraperitoneally. An additional control group of gerbils (n = 10) received PBS and complete Freund's adjuvant. On days 28 and 47, gerbils received a booster immunization with 150 μg of protein or PBS in incomplete Freund's adjuvant. Comparisons of vaccine efficacy were made with Fisher's exact test; two-tailed values are presented.

Serum was obtained from all gerbils immediately prior to primary vaccination (preimmune serum), on day 61 (prechallenge serum), and at the time of sacrifice (day 75). The immunogenicity of the recombinant proteins was analyzed by use of Western blotting or an enzyme-linked immunosorbent assay (ELISA) to measure the reactivity of serum (diluted 1:1,000) from immunized gerbils with *E. histolytica* HM1:IMSS lysates as previously described (18). Neither preimmune nor prechallenge serum from any gerbils immunized with GST, MBP, or PBS showed reactivity with *E. histolytica* HM1:IMSS trophozoite lysates in Western blotting or the ELISA (Fig. 1; data are not shown for GST- or PBS-immunized gerbils). Gerbils in each of the control groups (MBP, GST, and PBS groups) had antiamebic antibodies at the time of sacrifice (day 75) (Fig. 1), but the mean optical densities were significantly lower than those seen in serum samples obtained from SREHP/MBP-, 170CR/GST-, or 170/SR-immunized gerbils at either the prechallenge or the sacrifice time point.

Prechallenge serum from all gerbils immunized with 170CR/GST reacted with an amebic protein of 170 kDa in Western blot analysis, while prechallenge serum from all SREHP/MBP-immunized gerbils reacted with the 47- or 52-kDa native SREHP molecule. Prechallenge serum from all gerbils immunized with 170/SR reacted with both the native 170-kDa molecule and the native SREHP molecule (data not shown). All prechallenge and sacrifice serum samples (1:1,000 dilution) from gerbils immunized with recombinant *E. histolytica* antigens were reactive with amebic lysates when assessed in the ELISA (Fig. 1), and serum samples from 9 of 12 gerbils immunized with 170CR/GST, 5 of 12 gerbils immunized with SREHP/MBP, and 9 of 14 gerbils immunized with 170/SR were reactive with amebic lysates at a 1:10,000 dilution. A comparison of the mean ± standard deviation optical densities obtained for serum samples (1:1,000 dilution) that reacted with amebic lysates revealed no differences between the prechallenge and sacrifice values for each of the recombinant antigen-immunized groups. Hence, there was no evidence for a significant booster effect of live amebae in the SREHP/MBP-, 170CR/GST-, or 170/SR-immunized animals.

On day 68 (21 days following the final booster immunization), gerbils were challenged with an intrahepatic inoculation of 50,000 *E. histolytica* HM1:IMSS trophozoites as previously described (18). Seven days later the animals were killed, the entire liver was removed and weighed, and any abscess seen grossly was resected and weighed. The percentage of liver abscessed was calculated as the weight of the abscess divided

by the weight of the liver before abscess removal. Liver abscesses, as well as portions of randomly selected visually normal livers, were fixed in formalin, sectioned, and stained with hematoxylin and eosin for histological examination.

All animals vaccinated with PBS or MBP developed amebic liver abscesses, and 9 of 10 gerbils (90%) vaccinated with the GST fusion protein had amebic liver abscesses. The percentage of liver abscessed was not significantly different between the PBS-immunized controls (21% ± 16.3%) and either the MBP (20.3% ± 4.7%)- or the GST (10.4% ± 8.2%)-immunized gerbils. Most gerbils immunized with recombinant 170CR/GST were protected from amebic liver abscesses. Only 2 of 12 gerbils (17%) immunized with 170CR/GST developed amebic liver abscesses; this result was significantly different from the result obtained for GST-immunized gerbils ($P \leq 0.002$). The vaccine efficacy of 81% (in comparison with GST-immunized gerbils) to 83% (in comparison with PBS-immunized gerbils) is comparable to the 67% efficacy seen in gerbils immunized with the native 170-kDa protein (10). Amebic liver abscesses in the two gerbils that developed abscesses after 170CR/GST vaccination showed histopathology resembling that in control animals, with multiple granulomas, multinucleated giant cells, and a marked inflammatory infiltrate (data not shown). Because of the possibility that vaccination might have delayed abscess formation and immunized gerbils might have had microscopic foci of infection not visible by inspection of the liver at 7 days, we also examined sections from the livers of 170CR/GST-vaccinated gerbils with no visible abscesses. While there was inflammation in mesentery tissue adherent to the site of inoculation and some vacuolation in the cytoplasm of hepatic cells at the inoculation site, no amebae and few inflammatory cells were found in multiple sections. Similar results were obtained in the histopathologic examination of livers from SREHP/MBP-vaccinated gerbils and gerbils that were vaccinated with 170/SR and failed to develop amebic liver abscesses. The relative lack of pathology as early as 7 days after amebic inoculation in the livers of the recombinant *E. histolytica* antigen-vaccinated animals that did not have visible amebic liver abscesses would appear to be consistent with a model in which vaccination prevents the development of an amebic liver abscess rather than promoting the healing of an established abscess. However, a prospective trial examining vaccinated animals at earlier time points after hepatic inoculation with amebae will be necessary to resolve this question.

In this study we also confirmed the efficacy of immunization with SREHP/MBP in preventing amebic liver abscesses (18). All 12 gerbils immunized with recombinant SREHP/MBP failed to develop amebic liver abscesses, a result that was different from that obtained for the control, MBP-immunized group ($P < 0.0001$). The results obtained with SREHP/MBP immunization were not significantly different from the results obtained with 170CR/GST immunization ($P = 0.48$).

One possible strategy for a recombinant antigen-based vaccine used to prevent amebiasis would be the use of a multicomponent vaccine, containing a number of protective recombinant *E. histolytica* antigens in combination. However, combination vaccines could have increased toxicity and reduced efficacy. As an initial approach to this problem, we also immunized gerbils with 170/SR. The combination vaccine appeared to be tolerated as well as each individual component. Of 14 gerbils immunized with 170/SR, only 2 (14%) developed amebic liver abscesses after direct hepatic inoculation of amebic trophozoites. This result was significantly different from that obtained for GST-immunized animals ($P < 0.001$) but did not differ from that obtained with SREHP/MBP alone ($P = 0.48$) or 170CR/GST alone ($P = 1.0$). Given the similarity in the levels of protection seen with each *E. histolytica* recombinant antigen preparation, a prohibitively large trial would probably be necessary to detect differences in protective efficacy among the three recombinant vaccine preparations.

The current trial was not designed to investigate how vaccination protects against amebic liver abscess in the gerbil model. Studies using passive immunization of mice that have severe combined immunodeficiency with antiamebic antiserum or anti-SREHP antiserum have suggested a potential role for preexisting antibody in protection against amebic liver abscess (2, 17). However, in this study, as in our previous study of SREHP/MBP immunization (18), the prechallenge level of antibody titers against amebae did not correlate with protection in gerbils immunized with recombinant 170CR/GST or 170/SR (Fig. 1). A protective role for cell-mediated immunity was not addressed in this study, but this is a definite possibility, since both recombinant *E. histolytica* proteins appear to be capable of eliciting cell-mediated responses. Immunization with recombinant SREHP/MBP induced delayed-type hypersensitivity responses to SREHP in gerbils (18), and the native 170-kDa molecule can elicit an in vitro amebicidal cell-mediated response in lymphocytes from patients with a prior *E. histolytica* infection (14).

In summary, we have shown that immunization of gerbils with a recombinant version of a portion of the 170-kDa surface antigen can provide protection against amebic liver abscess which is at least equivalent to the level of protection obtained during immunization with the native molecule (10). In addition, we have demonstrated that a combination vaccine containing the 170CR/GST fusion protein and the SREHP/MBP fusion protein can be safely administered and provides a high level of protection in the gerbil model. Important questions regarding how long protective immunity persists after immunization, whether this protection will extend to other *E. histolytica* strains besides HM1:IMSS, whether parenteral vaccination provides any protection against intestinal disease, and how protection is mediated in this system remain to be answered. Nevertheless, the identification of protective recombinant antigens represents a step in the development of a subunit vaccine to prevent amebiasis.

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