Progress towards Development of a Vaccine for Amebiasis

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INTRODUCTION

The protozoan parasite Entamoeba histolytica is the causative agent of amebic dysentery and amebic liver abscess. It has been estimated that 50,000,000 people worldwide suffer from diarrhea secondary to E. histolytica infection and that amebic dysentery and amebic liver abscess kill at least 50,000 individuals yearly (117). The disease is far more prevalent in developing countries, and there appear to be regional hot spots for E. histolytica infection where morbidity and mortality from amebiasis seem especially high. Such regions include Mexico, South Africa, and India (117). The magnitude of the problem in Mexico has been illustrated by a recent nationwide serologic survey that showed that more than 8% of the Mexican population have had an episode of E. histolytica infection (10). While effective therapy for amebiasis is available in the form of the drug metronidazole, the use of this agent does not appear to have had any significant effect on the prevalence of amebic infection. The main mode of transmission of amebiasis is the ingestion of fecally contaminated food or water containing E. histolytica cysts; therefore, improved sanitary conditions are clearly the long-term approach to reducing or eliminating amebiasis from areas of endemic infection. However, it is unlikely that this will be accomplished in the near future. For this reason, several laboratories have initiated efforts towards developing a vaccine to prevent amebic infection. In this review, I will discuss the scientific rationale for an amebiasis vaccine, identify candidate E. histolytica antigens for inclusion in a vaccine, and summarize the recent progress made in developing and testing both parenteral and oral recombinant E. histolytica antigen-based vaccines for amebiasis.

E. HISTOLYTICA INFECTION

E. histolytica is a parasitic ameba of the human gastrointestinal tract. It has a simple life cycle, existing only as the motile trophozoite or the hardy, infective, nonmotile cyst stage (Fig. 1) (reviewed in reference 51). E. histolytica naturally infects only humans and perhaps some higher nonhuman primates. Infection is established by ingestion of the cyst stage of the parasite. After cysts pass through the stomach and the proximal small bowel, they excyst to the motile trophozoite form, which colonizes and can penetrate the colonic mucosa. Under still undefined conditions within the colon, trophozoites encyst, and the infective cysts are passed out in the stool, where they can infect new hosts. Trophozoites may be passed in the stool as well, but they are noninfectious. Invasion of the colonic mucosa by E. histolytica trophozoites results in diarrhea, which is usually bloody (amebic dysentery). In roughly 5 to 10% of individuals with intestinal amebiasis, the E. histolytica trophozoites invade through the colonic mucosa and reach the portal circulation, which carries them to the liver. There they can establish amebic liver abscess, a major cause of liver abscesses worldwide.

Until recently, one unexplained aspect of the epidemiology of amebiasis was the presence of a large number of asymptomatic individuals who passed cysts but had no evidence for invasive disease. Pioneering work involving isoenzyme analysis (85–87) and, more recently, molecular techniques (9, 14, 108, 111) showed conclusively that there are two genetically distinct but morphologically identical species of what was formerly referred to as Entamoeba histolytica. One species, which is clearly associated with disease in humans, is still referred to as Entamoeba histolytica. The second species, which is more prevalent but does not appear to be associated with disease in humans, is called Entamoeba dispar. The vaccine work reviewed here deals solely with E. histolytica, the agent of amebic dysentery and amebic liver abscess.

FEASIBILITY OF A VACCINE TO PREVENT AMEBIASIS

The challenges in developing an effective vaccine against any infectious disease are daunting, and parasitic diseases offer a number of additional problems. In many cases, protozoan parasites have complex life cycles with multiple hosts and stages.
chronic infection, such as malaria, appear to pose particular challenges for successful vaccine development (70). Data on the acquisition of immunity following infection with *E. histolytica* are limited. In one epidemiologic study, individuals with a prior episode of amebic liver abscess had a much lower rate (0.2% during the study) of contracting a liver abscess than did previously uninfected patients based on historical controls (22), suggesting that prior infection may result in protective immunity. The experience of clinicians in areas of endemic infection appears to support this concept (92), but carefully controlled prospective studies are simply not available.

If protective immunity can develop after amebic infection in humans, how is it mediated? One possible mechanism involves the development of anti-amebic antibodies. Individuals with amebic liver abscess develop anti-amebic antibodies, which are usually detectable within 1 week of the onset of symptoms (38, 43). These anti-amebic antibodies form the basis of serologic tests used to diagnose amebic liver abscess. The development of anti-amebic antibodies appears to have no effect on the course of amebic liver abscess, which has a high fatality rate without appropriate therapy (16). However, the failure of antibodies to alter the course of established amebic liver abscess does not mean that preexisting anti-amebic antibodies could not provide protection against subsequent infection (78). The lower inoculum associated with new infection, and antibody contact with trophozoites in the bloodstream rather than in abscess tissue, might explain the difference in efficacy. There are experimental studies to support this concept. Hamsters passively immunized with human immune serum and then intraperitoneally challenged with *E. histolytica* trophozoites were partially protected from amebic liver abscess (91). Recently, we demonstrated that human anti-amebic antibodies, obtained from patients with amebic liver abscess, could protect mice with severe combined immunodeficiency (SCID mice) from developing an amebic liver abscess when the antibodies were administered before an intraperitoneal challenge with virulent *E. histolytica* trophozoites (93). Because SCID mice lack functional B or T lymphocytes, these studies clearly indicate that preexisting human anti-amebic antibody alone can protect against amebic liver abscess in this model.

The production of mucosal immunoglobulin A (IgA) anti-amebic antibodies after invasive intestinal amebiasis could also play a role in protecting against subsequent intestinal infection with *E. histolytica*. Anti-amebic antibodies of the IgA subclass have been detected in saliva from patients with intestinal amebiasis (11), and it has been demonstrated that human salivary IgA anti-amebic antibodies can block *E. histolytica* adherence to a mammalian cell line, suggesting that they could play a role in inhibiting amebic infection/colonization in vivo (11). These findings speak directly to the feasibility of developing an oral vaccine for amebiasis that is capable of stimulating mucosal IgA anti-amebic antibodies that could block amebic adherence to human intestinal epithelial cells.

A role for cell-mediated immunity in protection against amebiasis has also been investigated. Individuals with amebic liver abscess and intestinal amebiasis develop T-cell proliferative responses to amebic antigens following infection (81, 82, 88, 115). When activated, macrophages from uninfected and previously infected individuals can kill amebic trophozoites in vitro (82, 83). It has also been reported that lectin-stimulated CD8+ T cells or amebic antigen-stimulated CD8+ T cells from patients treated for amebic liver abscess have cytotoxic activity against amebic trophozoites in vitro (81, 82); but how such killing occurs in the absence of class I antigen expression on the surface of *E. histolytica* trophozoites is unexplained. The strongest evidence for a role of cell-mediated immunity in

These create problems both in the logistics of working with the parasite and in the identification of protective antigens, since target antigens identified in one stage of the parasite may not be expressed in another stage, thus limiting their effectiveness as vaccine candidates. The presence of animal hosts for many parasites also limits the possibility of eradicating the disease by vaccination, since these alternative hosts may provide a constant reservoir for infection of humans. Most of the clinically important protozoan parasites are predominantly intracellular parasites, thus providing limited opportunities for antibody-mediated protection, a critical correlate of much vaccine-mediated immunity. Fortunately, as noted above, most of these problems do not apply to amebiasis. There are no insect vectors or secondary hosts involved in the transmission of amebiasis, and a vaccine that eliminated colonization and infection of humans with *E. histolytica* could eventually eradicate the disease and the organism. Finally, *E. histolytica* is an extracellular parasite, thus providing a potential role for antibody in vaccine-induced immunity to amebic infection.

An important consideration in evaluating the feasibility of a vaccine for amebiasis is whether immunity to subsequent infection develops following a bout of amebiasis. Those diseases for which protective and long-lived immunity develops following infection, such as mumps and smallpox, have been very effectively prevented by vaccines, while diseases for which the host immune response to initial infection is only minimally effective or ineffective in preventing subsequent infection or...

FIG. 1. Life cycle of *E. histolytica*. Disease begins when the cyst form of the parasite is ingested, excystation into the trophozoite form probably occurs in the lower small bowel, and the trophozoites are capable of penetrating into colonic mucosa as indicated. Trophozoites can encyst in the colon, and these cysts are excreted in the stool, releasing infectious cysts into the environment to renew the life cycle. In some cases, trophozoites invade through the colonic mucosa and reach the portal circulation, where they penetrate into liver tissue and create an amebic liver abscess.
protection from amebiasis comes from anecdotal reports of worsening complications of amebiasis in patients receiving corticosteroids (37, 57). However, arguing against a critical role for cell-mediated immunity in protection against amebiasis has been the absence to date of reports of any significant increase in the incidence of intestinal amebiasis or amebic liver abscess in patients with AIDS in areas where both diseases are endemic. Thus, while findings from patients indicate that cell-mediated immune responses to amebic antigens develop following infection, the role of these responses in protection against amebiasis in humans remain unknown, and whether a vaccine to prevent amebiasis must stimulate cell-mediated immune responses for protective efficacy is unclear.

Data obtained from animal studies of protective immunity to amebiasis indicate that immunity to amebiasis can be produced by prior infection or vaccination. Dogs reportedly develop immunity to reinfection after experimentally induced intestinal amebiasis, and passive immunization of naive dogs with sera from *E. histolytica*-immune animals refractory to amebic infection resulted in a decrease in susceptibility to intestinal amebiasis in the naive group (103). Following a drug-terminated bout of amebic liver abscess, hamsters were protected from a second infection (114). Guinea pigs infected intracecally with a noninvasive strain of *E. histolytica* were protected against subsequent challenge with a virulent strain of *E. histolytica* (34). In a number of different studies, rats, guinea pigs, hamsters, or monkeys vaccinated with either intact amebae or various forms of complex amebic antigens have been protected against amebic liver abscesses, or, in the case of guinea pigs, intracecal infection with *E. histolytica* trophozoites (31, 32, 35, 44, 56, 90, 116).

**PARENTERALLY ADMINISTERED RECOMBINANT ANTIGEN-BASED AMEBIASIS VACCINES**

There are a number of potential approaches to an amebiasis vaccine. To date, work has focused on the trophozoite stage of the parasite, since it can be cultivated in vitro, while no attempts to incorporate any antigens derived from the cyst stage (which cannot be obtained in culture) have yet been attempted. One approach, which has been successful for other pathogens, is to attempt to use killed or inactivated *E. histolytica* trophozoites as the vaccine components. This strategy has been successful in some of the animal studies cited above. However, it is relatively expensive to grow *E. histolytica* in culture, and the cost of producing *E. histolytica* trophozoites or antigens on the scale necessary for vaccine use could be prohibitive. In addition, there are always concerns about reactogenicity when using killed or inactivated whole organisms in vaccine preparations. For these reasons, much of the recent progress in a vaccine for amebiasis has come from the use of recombinant *E. histolytica* antigens. Recombinant techniques offer the possibility of large-scale production of structurally defined *E. histolytica* antigens through prokaryotic or yeast expression systems. In addition, they provide the opportunity to genetically engineer multiple antigen vaccines or to use attenuated viral or bacterial vectors as carriers for amebic antigens. There are now three different *E. histolytica* proteins that have formed the basis for recombinant antigen vaccines, the serine-rich *E. histolytica* protein (SREHP), the Gal/GalNAc binding lectin, and a 29-kDa antigen. The three subsequent sections will review what is known about the structure, function, and immunogenicity of each of these amebic antigens and the vaccine-related studies associated with them.

**Serine-rich *E. histolytica* Protein**

A partial cDNA clone encoding the SREHP was first isolated by differential screening with *E. histolytica* HM1:IMSS mRNA or mRNA from *Entamoeba moshkovskii* Laredo, a nonpathogenic species, of duplicate filters of a cDNA library derived from the virulent *E. histolytica* HM1:IMSS strain. This strategy was designed to isolate *E. histolytica*-specific genes that could play a role in virulence (97). Those *E. histolytica* cDNA clones hybridizing only with *E. histolytica* mRNA and not with both *E. histolytica* and *E. moshkovskii* mRNA were selected for further analysis. The full-length SREHP cDNA clone encodes a peptide of approximately 25 kDa, but the native SREHP molecule was found to migrate at 47 and 52 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This discrepancy is due both to posttranslational modifications of the SREHP molecule (101) and to the high hydrophilicity and multiple proline residues in the SREHP peptide which cause anomalous migration in SDS-PAGE (97). The protein was designated serine-rich *E. histolytica* protein due to the large number of serine residues (52 of 233 amino acids) (97). The other striking feature of the structure was the presence of multiple tandemly repeated octapeptides and dodecapeptides. Subsequently, a similar clone was independently isolated by antibody screening of an *E. histolytica* cDNA library, and it was found that *E. dispar* has a highly homologous gene with nucleotide differences between the *E. histolytica* and *E. dispar* SREHP cDNA sequences confined primarily to the tandem repeats (42). The structural motif of the SREHP molecule is somewhat reminiscent of the circumsporozoite proteins of malaria, with a hydrophobic N-terminal region (signal sequence) followed by a region of charged amino acids at the N terminus, the presence of multiple tandem repeats, posttranslational modifications which include multiple O-linked phosphate residues on serines, O-linked glucosamine residues, and the presence of a hydrophobic domain at the C terminus, which is probably anchored in the plasma membrane by an ester-linked lipid group.

**FIG. 2. Model of the native SREHP.** Pertinent features of the SREHP molecule are indicated, including a region of charged amino acids at the N terminus, the presence of multiple tandem repeats, posttranslational modifications which include multiple O-linked phosphate residues on serines, O-linked glucosamine residues, and the presence of a hydrophobic domain at the C terminus, which is probably anchored in the plasma membrane by an ester-linked lipid group.
of serine residues and glycosylation with O-linked N-acetylglucosamine residues, which are more commonly seen in nuclear or cytoplasmic proteins (101). It appears to be anchored in the membrane by a C-terminal covalently linked lipid (101). The function of SREHP remains unclear. Antibodies to SREHP block amebic adherence to mammalian cells in vitro, but the isolated SREHP molecule shows no obvious adhesin properties (97, 121). Recently, we demonstrated that SREHP can serve as a chemoattractant for *E. histolytica* trophozoites, but the physiologic relevance of this property is unknown (101).

SREHP is highly immunogenic and appears to possess a number of conserved epitopes. Among individuals with amebic liver abscess, more than 80% have antibodies that recognize the SREHP molecule (100). SREHP appears to be the product of a single-copy gene (50), and while the SREHP genes from different isolates of *E. histolytica* can differ in the number of dodecapeptide and octapeptide repeats encoded (this property is the basis of PCR tests that can differentiate among strains of *E. histolytica* [15]), no strain completely lacking sequences encoding the dodecapeptide repeat of SREHP has been described. Consistent with this finding, when eight different clinical and laboratory isolates of *E. histolytica* and *E. dispar* were analyzed by immunoblotting with a monoclonal antibody that recognizes the dodecapeptide repeat of SREHP, all the strains analyzed possessed a protein product that reacted with the monoclonal antibody, although there was size variation in this protein among the strains (96). Epitope mapping with serum from patients with amebic liver abscess suggests that most antibodies to SREHP bind epitopes within the dodecapeptide and octapeptide repeats (118).

The aforementioned properties of surface location, the ability of SREHP immunization to induce amebic adherence blocking antibodies, and the presence of conserved SREHP epitopes in geographically diverse isolates of *E. histolytica* all suggested that SREHP could be a viable vaccine candidate. Further support for testing SREHP as a parenteral vaccine to prevent amebic liver abscess came from passive immunization studies. SCID mice were passively immunized with rabbit polyclonal antisera to recombinant SREHP-TrpE and then intrahepatically challenged with virulent *E. histolytica* HM1:IMSS trophozoites. None of the SCID mice receiving the anti-SREHP antibodies developed an amebic liver abscess, while all the SCID mice receiving preimmune serum developed an abscess (121). These data established the efficacy of anti-SREHP antibodies in protecting against amebic liver abscess and provided some of the strongest evidence for a protective role of preexisting anti-amebic antibodies in preventing the development of amebic liver abscess.

The SREHP cDNA has been expressed as TrpE, glutathione-S-transferase (GST), and maltose binding protein (MBP) fusion proteins in *Escherichia coli* (62, 97). Expression of SREHP in *E. coli* without a fusion partner has not been achieved, suggesting that SREHP alone may be toxic for bacterial cells. SREHP has been expressed without any fusion partner in baculovirus (101), where recombinant SREHP reaches the surface of virally infected insect cells and is phosphorylated and glycosylated but does not appear to have a lipid anchor (101). The highest yield of soluble recombinant SREHP was obtained with MBP as the fusion partner, and this was the preparation used for parenteral vaccine studies (62).

To test the safety and protective efficacy of SREHP-MBP vaccination, a gerbil model of amebic liver abscess was used. Gerbils develop amebic liver abscess after direct intrahepatic inoculation with virulent amebic trophozoites, and if untreated, they will usually die of the infection in 21 days (12). In two separate trials, gerbils were vaccinated with three doses of SREHP-MBP intraperitoneally, while in another trial, a single dose of SREHP-MBP was administered intradermally. Control groups received equivalent doses of MBP or phosphate-buffered saline (PBS) (122, 124). Vaccination was well tolerated by all groups. Approximately 3 weeks after vaccination, all the gerbils were challenged intrahepatically with virulent *E. histolytica* HM1:IMSS trophozoites, and 7 days later the animals were sacrificed and liver abscess formation was assessed. The readout in these studies was whether an abscess could be detected at 7 days and the percentage (based on weight) of the liver occupied by an amebic abscess in animals developing disease. Among gerbils vaccinated with SREHP-MBP in the three trials, the protective efficacy of vaccination (no amebic liver abscess detectable) was 64% of vaccinated animals in trial 1, 100% of vaccinated animals in trial 2 (intradermal vaccination), and 100% of vaccinated animals in trial 3 (122, 124). In contrast, all MBP- or PBS-vaccinated gerbils developed amebic liver abscess (protective efficacy of 0%). SREHP-MBP-vaccinated gerbils developed antibody and cell-mediated immune responses (as measured by delayed-type hypersensitivity) to SREHP and ameba. Quantitatively similar anti-amebic antibody responses were seen in gerbils that were protected from amebic liver abscess and in the few SREHP-MBP vaccine failures seen in these studies, making it difficult to ascribe protective immunity solely to the magnitude of the anti-SREHP antibody response. Whether there are differences in the epitopes recognized in the anti-SREHP response in vaccine successes and failures was not examined in this study.

The high level of complete protection from amebic liver abscess seen in gerbils vaccinated with recombinant SREHP-MBP demonstrated the potential of SREHP as a vaccine candidate and illustrated the feasibility of using recombinant *E. histolytica* antigens in amebiasis vaccines. Subsequently, a preliminary analysis of the safety and efficacy of the recombinant SREHP-MBP vaccine in nonhuman primates was performed (98). Two African green monkeys were vaccinated with SREHP-MBP in three doses on days 0, 30, and 53 by subcutaneous administration; a single control monkey received PBS and adjuvant alone at the same time points. The SREHP-MBP vaccine was well tolerated, with no systemic side effects detected in the vaccinated monkeys and with no abnormalities in liver function tests, complete blood count, or renal function seen in the vaccinated monkeys or the single control animal. Some local induration at the inoculation site was seen in both the control monkey and the SREHP-MBP-vaccinated animals; this was believed to be secondary to the Freund adjuvant used in these experiments. At 10 days following the first booster vaccine dose, anti-SREHP and anti-amebic antibodies were detected in SREHP-MBP-vaccinated monkeys; anti-SREHP and anti-amebic antibody titers did not significantly increase following the second booster dose of SREHP-MBP. The single control monkey had no anti-amebic or anti-SREHP antibodies. Importantly, serum from SREHP-MBP-vaccinated animals obtained after the first or second booster immunization could significantly inhibit amebic adherence to Chinese hamster ovary (CHO) cells in vitro (98). The ability of anti-amebic serum to inhibit amebic adherence is the property that correlated with protection from amebic liver abscess in the passive immunization studies in SCID mice (121). A major disappointment in this trial, and the reason why the study was limited to a few monkeys, was the inability to test the protective efficacy of SREHP-MBP vaccination in African green monkeys (98). Contrary to a published report, in this study amebic liver abscesses could not be established in *E. histolytica*-naive (based on amebic serology and stool examination for amebic trophozoites) African green monkeys by direct hepatic inoculation.
with virulent amebic trophozoites, eliminating the possibility of studies of protective efficacy in this primate species. Despite this limitation, demonstrating the safety and immunogenicity of the SREHP-MBP preparation in nonhuman primates represented an important step in the development of a recombinant SREHP-based amebiasis vaccine.

**E. histolytica Gal/GalNAc-Specific Lectin**

One of the best-studied molecules of *E. histolytica* is a surface adhesion with specificity for galactose and N-acetylgalactosamine residues, designated the Gal/GalNAc lectin (reviewed in reference 58). The adhesin is large (260 kDa) and can be separated into component heavy (170-kDa) and light (35- or 31-kDa) subunits by SDS-PAGE under reducing conditions (66). The primary structures of both the heavy and light chain subunits have been obtained from the nucleotide sequence from cDNA clones and genomic sequences (55, 59, 71, 109). The derived amino acid sequence of the heavy (170-kDa) subunit contains a signal sequence, an approximately 370-amino-acid cysteine-rich region, an approximately 275-amino-acid region of pseudorepeats of 30 amino acids containing four conserved cysteine residues, an approximately 550-amino-acid cysteine-rich region (10.9% cysteine residues) domain, a 26- to 28-amino-acid putative hydrophobic transmembrane domain, and a cytoplasmic tail of approximately 40 amino acids (55, 109). The 170-kDa subunit is encoded by at least three genes, with a predicted mass of 32 kDa, which may be anchored to the membrane by a glycosylphosphatidylinositol anchor (59, 110). At least two light-subunit genes are present and encode proteins with derived amino acid sequences that are 80.6% identical (59, 60, 110).

The Gal/GalNAc lectin appears to play an important role in amebic binding to mammalian cells. The native adhesin binds to galactose and can be affinity purified by galactose-Sepharose (68). *E. histolytica* binding to CHO cells can be blocked by galactose, implicating the Gal/GalNAc lectin in amebic adherence to target cells (72). In addition, studies with mutant CHO cells have shown that *E. histolytica* trophozoites show greatly reduced binding to mutant cells lacking terminal galactose or N-acetylgalactosamine residues (48, 75). Amebic cytolysis of mammalian cells is contact dependent, and the mutant CHO cells lacking terminal N-acetylgalactosamine residues are partially resistant to killing by *E. histolytica* trophozoites (49). Monoclonal antibodies directed against certain regions of the Gal/GalNAc lectin can inhibit amebic adherence to CHO cells (74), while monoclonal antibodies to other epitopes actually enhance amebic adherence to the same cells (69). All monoclonal antibodies to the Gal/GalNAc lectin that affect adherence appear to bind to the cysteine-rich region of the heavy subunit (69, 74). The Gal/GalNAc lectin has also been implicated in the ability of *E. histolytica* trophozoites to resist killing by human complement. An anti-amebic monoclonal antibody that renders *E. histolytica* trophozoites susceptible to killing by human complement components C5b9 was found to bind epitopes within the cysteine-rich region of the 170-kDa subunit of the Gal/GalNAc lectin (7). This region of the 170-kDa subunit showed some homology to the human complement regulatory protein CD59, a molecule which can block assembly of the C5b-9 attack complex, thus providing a potential mechanism for complement resistance by amebae. What remains unclear is whether naturally occurring complement-resistant and complement-susceptible *E. histolytica* isolates differ in either the primary structure in the CD59-like region of the 170-kDa molecule or the surface expression of the Gal/GalNAc lectin.

The Gal/GalNAc lectin is highly immunogenic, with more than 90% of patients with amebic liver abscess developing antibodies capable of binding to the native molecule (73). Antibodies appear to be directed almost entirely against the 170-kDa subunit (73). There are significant differences in the immunogenicity of the three structural domains of the 170-kDa subunit. Using recombinant GST fusion proteins containing the entire 170-kDa sequence (except the signal sequence and the putative transmembrane and cytoplasmic domains), the cysteine-poor region alone (designated 170CP), the pseudorepeat region alone (designated 170PR), and the cysteine-rich region alone (designated 170CR), we found that all the patients with amebic liver abscess, who came from several different geographic regions, had antibodies to the entire 170-kDa molecule and that they all had antibodies that bound to the cysteine-rich region (170CR fusion protein) (127). Approximately 89% of the patients with amebic liver abscess had antibodies to the cysteine-poor domain of the molecule (170CP fusion protein), while only 9% had antibodies that bound to the pseudorepeat region (170PR fusion protein). The reason for the poor immunogenicity of the 170PR region is unknown but could reflect MHC restrictions or the induction of antibodies that recognize conformational epitopes and hence do not react with the denatured 170PR fusion protein. The immunogenic properties of the cysteine-rich region of the 170-kDa subunit were confirmed and extended by recent studies demonstrating that more than 95% of sera from patients with amebic liver abscess have IgA antibodies that recognize a recombinant protein derived from the cysteine-rich domain of the 170-kDa subunit (94) and that more than 80% of individuals with amebic colitis develop mucosal IgA antibodies that bind this recombinant protein (1).

The function of the Gal/GalNAc lectin in amebic adherence, the potential role of this molecule in mediating *E. histolytica* resistance to complement, the immunogenicity of the heavy subunit, and the presence of highly conserved epitopes made this molecule an obvious vaccine candidate. The first vaccine studies were performed with the purified native 220-kDa Gal/GalNAc lectin and the gerbil model of amebic liver abscess. The purified Gal/GalNAc lectin was administered as a primary dose and two booster doses with Freund's adjuvant (67). Gerbils vaccinated with the purified lectin developed antibodies that recognized the 170-kDa subunit of the molecule; antibodies to the light subunit were not detected. Serum from vaccinated gerbils blocked amebic adherence to CHO cells when used at a 1/10 dilution but enhanced adherence when used at a 1/1,000 dilution (67). Among control animals receiving only the adjuvant, 81% developed an amebic liver abscess, while in the Gal/GalNAc-vaccinated gerbils, only 27% developed an amebic liver abscess, representing a 67% reduction in the incidence of liver abscess in the vaccinated gerbils (67). One unexpected finding was that the amebic liver abscesses detected in gerbils vaccinated with the Gal/GalNAc lectin were significantly larger than those seen in the control gerbils. This study established the vaccine potential of the Gal/GalNAc lectin and demonstrated that vaccination with a purified amebic antigen could provide protection against amebic liver abscess. It also suggested that some component of the immune response to vaccination with the Gal/GalNAc lectin could, in a subgroup of gerbils, exacerbate amebic liver abscess.

The success of the native Gal/GalNAc lectin in vaccine studies provided a rationale for exploring the efficacy of recombi-
nant Gal/GalNAc lectin antigens in protection against amebic liver abscess. In addition, it was anticipated that the ability to use only the heavy subunit of the lectin, and even specific domains of that molecule, might allow one to develop a recombinant Gal/GalNAc-based vaccine that could mimic the protective effects of the native molecule without any exacerbative effects on amebic liver abscess. Based on the serologic mapping studies, which showed that all patients with amebic liver abscess had antibodies that recognized epitopes on the cysteine-rich domain of the 170-kDa subunit, and the studies which suggested that both the carbohydrate-binding domains of the molecule and the region of the molecule involved in complement resistance map to the cysteine-rich region, we selected a GST fusion protein containing this domain (170CR) for the initial vaccine studies (124). Gerbils received a primary immunization and two booster immunizations with the 170CR protein (which contained amino acids 649 to 1202 of the 170-kDa subunit of the Gal/GalNAc lectin fused to GST) in Freund’s adjuvant and were challenged intrahepatically with virulent E. histolytica trophozoites 21 days after the second booster dose. Control gerbils were immunized with the GST fusion protein alone and adjuvant or with PBS and adjuvant. Among gerbils vaccinated with 170CR, only 17% developed an amebic liver abscess compared with 100% of PBS and adjuvant-vaccinated gerbils and 90% of GST- and adjuvant-vaccinated gerbils. The protective efficacy of the recombinant 170CR vaccine was 81%, a figure that compares well with the 67% efficacy seen with vaccination with the native Gal/GalNAc lectin (67). In addition, the few vaccine “failures” in this study, i.e., gerbils that were vaccinated with the 170CR protein but did not develop an amebic liver abscess, did not have abscesses that were larger than those in either the GST-vaccinated or PBS-vaccinated control groups (124). These data indicated that the recombinant 170CR vaccine could perform as well or better than the native Gal/GalNAc lectin-based vaccine in protection against amebic liver abscess and might offer a significant advantage by not exacerbating disease in vaccine failures. Independent confirmation of these findings came from a subsequent study in which another cysteine-rich region-based recombinant protein (this time containing amino acids 758 to 1202 of the 170-kDa subunit of the Gal/GalNAc lectin fused to GST) in Freund’s adjuvant and two booster immunizations with the recombinant 170CR protein developed an amebic liver abscess, did not have abscesses versus 100 and 90% of MBP- and GST-vaccinated gerbils, respectively (124). Comparison with SREHP-MBP alone or 170CR alone did not show increased efficacy for the combination vaccine, however, and if trials were continued under similar conditions, it was estimated that a prohibitive number of animals would have to be used to show a difference in protective efficacy among the three different vaccine preparations (124). Whether the SREHP-MBP–170CR combination vaccine would prove superior to the single-vaccine preparations if a higher challenge dose of amebae was used or would allow reduced dosing of each of the vaccine components remains untested. However, these data indicate that simultaneous administration of the SREHP-MBP and 170CR vaccines does not appear to diminish the immune response to either antigen, and they suggest that a combination vaccine containing those two antigens is feasible.

29-kDa Cysteine-Rich E. histolytica Antigen

A partial cDNA clone encoding a 29-kDa cysteine-rich amebic antigen was first identified by anti-amebic antibody screening of an E. histolytica strain H-302:NIH cDNA library (113). The derived amino acid sequence showed 7% cysteine residues with multiple cysteine doublets (113). Analysis of a full-length cDNA and genomic clones showed that the derived amino acid sequence of the 29-kDa protein has significant relatedness to the sequences of hydrogen peroxide-inactivating proteins that are found in gut-living prokaryotes (8). These findings were of interest because it had been previously shown that E. histolytica strains lack catalase and peroxidase but possess superoxide dismutase, raising the question of how H$_2$O$_2$ is detoxified by amebae (8, 107). Attempts to localize the 29-kDa cysteine-rich protein in E. histolytica have given differing results. The putative function of this molecule, the absence of a signal sequence, and indirect immunofluorescence studies have been used to argue that the 29-kDa antigen is located in the cytoplasm and nucleus of E. histolytica trophozoites (8, 104). However, surface labeling, indirect immunofluorescence, and transmission electron microscopy with immunogold suggest that the 29-kDa antigen can be found on the surface membrane of amebae (28, 77, 113). In this regard, it should be noted that the E. histolytica alcohol dehydrogenase 2 (EhADH2) molecule of E. histolytica, a critical enzyme in the amebic fermentation pathway, also lacks a signal sequence but that it (or immunologically identical isoforms) appears to be present both in the cytoplasm and on the surface membrane of E. histolytica trophozoites (30).

The 29-kDa molecule is immunogenic. Approximately 80% of individuals with amebic liver abscess develop antibodies that bind to the native or recombinant 29-kDa protein (29, 95). A recombinant 29-kDa fusion protein derived from the sequence of a full-length cDNA clone fused to sequences encoding six histidine residues was used for the first vaccine trials of this antigen (95). Gerbils received three immunizations with the recombinant protein and Titermax adjuvant at 2-week intervals and then were challenged with an intrahepatic inoculation of E. histolytica trophozoites some time following the final booster dose. Control groups of gerbils received three immunizations with Titermax or saline alone. Gerbils vaccinated with the recombinant 29-kDa fusion protein developed antibodies that recognized the native 29-kDa protein in amebic lysates, while sera from control gerbils showed no reactivity with adjuvant. Vaccinated gerbils developed antibodies that recognized both the 170-kDa subunit of the lectin and the native SREHP molecule. Gerbils receiving the combination vaccine were protected against amebic liver abscess, with only 14% of vaccinated gerbils developing amebic liver abscess versus 100 and 90% of MBP- and GST-vaccinated gerbils, respectively (124).
with the native molecule. Among gerbils vaccinated with the recombinant 29-kDa protein, 30% developed an amebic liver abscess, while in the Titermax control group, 59% developed an abscess, for a calculated vaccine efficacy of 49%.

The protective efficacy of 49% seen with the recombinant 29-kDa antigen is lower than that reported with the recombinant SREHP-MBP, 170CR, or LC3 antigen preparations (94, 122, 124). However, differences in the number of E. histolytica trophozoites used for liver inoculation, the virulence of the ameba used, and the dose of vaccine administered make comparisons between the SREHP-MBP and 170CR trials and the recombinant LC3 and 29-kDa antigen trials problematic. However, the LC3 and 29-kDa antigen trials were performed under identical conditions, and the results with their control groups were identical (or the same control group was used for each trial), making the comparisons valid. When the results are directly compared, there is no statistically significant difference in protective efficacy between the recombinant 29-kDa antigen and the LC3 preparation ($P > 0.5$).

Future Vaccine Candidates

The recombinant SREHP, 170-kDa subunit of the Gal/GalNAc lectin, and 29-kDa antigen preparations and the native 220-kDa Gal/GalNAc protein are the only defined amebic antigens tested as vaccines in animal models of amebic liver abscess. However, there are a number of other E. histolytica antigens that represent potential candidates for inclusion in an amebiasis vaccine. The neutral amebic cysteine protease is a recognized virulence factor of E. histolytica and appears to play a critical role in amebic invasion and destruction of host extracellular matrix tissues (39, 76, 112). Patients who have had amebic liver abscess make antibodies that recognize amebic cysteine protease (65), and neutralization of the effects of cysteine protease by protease inhibitors can reduce the size of amebic liver abscess in SCID mice (102). A recombinant version of the 27-kDa E. histolytica cysteine protease I (EhCP1) has been produced. However, the recombinant EhCP1 protein lacked protease activity, probably because it failed to achieve the conformation of the native protease (102). Whether the presumed conformational difference between the recombinant protein and the native cysteine protease would compromise the efficacy of the recombinant EhCP1 molecule as a vaccine remains to be tested.

Another potential vaccine candidate is amebapore, a peptide of 77 amino acids that appears to play an important role in E. histolytica pathogenesis (reviewed in reference 47). This pore-forming protein forms ion channels in lipid bilayers and probably plays a critical role in the cytolytic activity of E. histolytica trophozoites (47). Synthetic peptides derived from the sequence of amebapore have been produced, and a peptide that can reproduce the cytolytic and pore-forming properties of the native protein have been identified (2, 46). Chemically produced isoforms of amebapore, which lack cytolytic and pore-forming activity based on the modification of histidine or lysine residues, have been generated (3); if these compounds are immunogenic and if antibodies to them neutralize the cytolytic activity of the native amebapore, the inactive isoforms could serve as the basis for an amebapore-derived vaccine. Other potential candidates include the 96-kDa E. histolytica EhADH2, a bifunctional enzyme with alcohol dehydrogenase and acetaldehyde dehydrogenase activities that plays a key role in the amebic fermentation pathway and is implicated in amebic adherence to extracellular matrix proteins such as fibronectin and laminin (119). A recombinant version of the EhADH2 protein has been produced in E. coli and did not require a fusion partner (120). The amebic glycoconjugate is a highly abundant surface molecule that structurally resembles the Leishmania lipophosphoglycan molecule (99). The amebic glycoconjugate is highly immunogenic, and antibodies to this molecule can block amebic adherence to mammalian cells (99). However, there has not yet been an identifiable peptide component to this molecule, and the immunogenic epitopes are probably carbohydrates, indicating that the native molecule would probably be required for any vaccine studies.

Limitations of Current Vaccine Studies

The studies cited above have demonstrated the feasibility of developing recombinant parenteral vaccines to prevent amebic liver abscess and have identified three recombinant amebic antigens that can protect in the gerbil model of disease. However, there are still a number of important issues to be resolved in the recombinant amebic antigen vaccine studies. First, all the studies to assess protection from amebic liver abscess after vaccination have been done in one animal model, the gerbil, and use an artificial form of establishing amebic liver abscess, direct hepatic inoculation of trophozoites. While it could be argued that this is a more stringent challenge than the natural route in humans (i.e., hematogenous spread of what is probably significantly fewer amebae into the liver), the predictive value of the gerbil model for vaccine success in humans remains unknown. Second, the cDNA sequences for the recombinant antigens used in these studies were derived from the HM1:IMSS laboratory strain of E. histolytica, and the same strain, HM1:IMSS, was used as the challenge organism in all studies. While there is ample serologic evidence to suggest that there are highly conserved epitopes in the SREHP, 170-kDa Gal/GalNAc binding lectin, and 29-kDa antigens (29, 73, 94, 95, 100, 127), no data have yet been presented to demonstrate that the HM1:IMSS-derived recombinant antigens can protect gerbils against “wild-type” E. histolytica isolates. Also unresolved is the question of the duration of protective immunity in recombinant amebic antigen-vaccinated gerbils. The amebic challenges have been performed at time points (1 to 3 weeks following booster immunizations) when circulating anti-amebic antibodies are detectable; whether protection would be seen at much later time points (e.g., 6 months) after vaccination needs to be studied. Questions regarding the optimum vaccine dosing and interval, as well as the most effective adjuvants, also remain unanswered. The critical question whether parenteral immunization with any of the candidate vaccine antigens provides protection against intestinal amebiasis has also not been approached, due to the lack of reliable animal models for intestinal disease. Finally, how protection is mediated in the gerbil model of amebic liver abscess remains unclear. A protective role for anti-amebic antibodies could be assessed by measuring the protective efficacy of passive immunization of naive gerbils with antibodies from recombinant amebic antigen-vaccinated gerbils. However, the lack of gerbil-specific monoclonal antibody reagents for T-cell markers or cytokines makes an assessment of the contribution of cell-mediated immunity to vaccine-induced protection difficult.

DEVELOPMENT OF AN ORAL VACCINE TO PREVENT AMEBIASIS

Amebiasis begins as an intestinal infection, and the ability of E. histolytica trophozoites to adhere to intestinal epithelial cells
may be critical in maintaining colonization and initiating disease (reviewed in reference 51). Mucosal anti-amebic antibodies could play a critical role in host defense, by blocking the ability of *E. histolytica* trophozoites to attach to the intestinal surface. Thus, a vaccine capable of stimulating mucosal IgA anti-amebic antibodies which block amebic adherence to intestinal epithelial cells could provide protection against both *E. histolytica* colonization and disease. Experimental support for this hypothesis has come from the aforementioned experiments showing that IgA antibodies isolated from the saliva of patients with amebic intestinal disease can block amebic adherence to target cells (11). In addition, it has been demonstrated that rats vaccinated with the native 220-kDa Gal/GalNAc lectin and the B subunit of cholera toxin, administered by direct inoculation into the rat Peyer’s patches, develop mucosal IgA anti-Gal/GalNAc antibodies capable of partially inhibiting amebic adherence to mammalian cells (41).

The best studied approach to stimulating mucosal IgA responses has been oral immunization. In addition to the advantage of potentially stimulating mucosal immune responses, oral vaccines may have significant advantages in acceptability, based on lower costs and ease of administration. To date, two different approaches to an oral vaccine for amebiasis have been used. The first approach is based on the oral administration of recombinant amebic antigens in conjunction with cholera toxin or the B subunit of cholera toxin, and the second approach is centered on the expression and delivery of recombinant amebic antigens by attenuated *Salmonella* or *Vibrio cholerae* carriers.

**Delivery of Recombinant *E. histolytica* Antigens with Cholera Toxin or Cholera Toxin B Subunit**

The rationale for combining amebic antigens with cholera toxin for oral immunization derives from the recognized adjuvant properties of cholera toxin. Cholera toxin is composed of one A subunit containing the active toxin domain (A1) and a short sequence (A2) which serves to link the A subunit non-covalently to five B subunits (reviewed in reference 5). The five B subunits mediate the binding of the toxin to *G*M₁ ganglioside on the surface of intestinal cells; this binding permits internalization of the A subunit into cells with resultant intoxication (17). Cholera toxin is a potent mucosal immunogen, inducing secretory IgA and serum IgA anti-toxin antibodies as well as serum IgG responses in animals when administered orally (27). Cholera toxin can also serve as a potent mucosal adjuvant. Proteins such as keyhole limpet hemocyanin (KLH) are normally not immunogenic when administered orally. However, when KLH is administered orally with cholera toxin, both anti-cholera toxin and anti-KLH antibodies are induced (26, 27, 53). The mechanism underlying the adjuvant properties of cholera toxin has been controversial, with some studies suggesting that toxin activity is required for adjuvant activity and others suggesting that the nontoxic B subunit can provide adjuvant activity (4, 20, 23, 33, 45, 52, 54, 61, 79, 106). There may be interspecies differences in the cholera toxin response, with mice requiring the toxin moiety for adjuvant activity while in humans the B subunit alone may serve as an adjuvant (21, 84). These points bear directly on the clinical utility of cholera toxin as an adjuvant, since administration of the nontoxic B subunit as an adjuvant is clearly preferable to a formulation with toxin activity.

Two recent studies have used cholera toxin B (CtxB) or the holotoxin as an adjuvant to facilitate the mucosal response to recombinant amebic antigens administered by the oral route. Using constructs developed by Dertzbaugh et al. (24, 25), we engineered a fusion protein (CtxB–SREHP-12) that contained the dodecapeptide repeat of SREHP fused to the cholera toxin B subunit (Fig. 3) (123). The CtxB–SREHP-12 protein could be purified from *E. coli* lysates by a two-step procedure, and, importantly, the CtxB moiety maintained the ability to bind to *G*M₁ ganglioside. In fact, inhibition studies showed that the CtxB–SREHP-12 molecule was as potent as CtxB alone in blocking the binding of labeled CtxB to *G*M₁ ganglioside. These results confirmed that small peptide molecules of at least 12 amino acids can be fused to the CtxB molecule without inhibiting the ability of CtxB to pentamerize and bind to *G*M₁ ganglioside. Mice were orally immunized with CtxB–SREHP-12 and a subclinical dose of cholera toxin on days 0, 14, and 28 and were sacrificed 7 days following the final boost, while control mice were orally vaccinated with CtxB and the subclinical dose of cholera toxin with the same schedule. At the time of sacrifice, the serum, bile, and stool were assayed for both IgG and IgA anti-amebic and anti-CtxB antibodies. In addition, the number of cells secreting IgA or IgG antibodies against SREHP, amebic lysates, cholera toxin, and cholera toxin B was assayed in samples obtained from the mesenteric lymph nodes and spleen. CtxB–SREHP-12-vaccinated mice developed IgA anti-amebic antibodies in stool and in bile. They also had significant numbers of antibody-secreting cells producing anti-SREHP and anti-amebic antibodies in mesenteric lymph nodes and spleen. In contrast, CtxB-vaccinated mice showed no level of anti-SREHP or anti-amebic antibodies or antibody-secreting cells that differed from background levels in any assay. Both CtxB- and CtxB–SREHP-12-vaccinated mice produced anti-CtxB antibodies, suggesting that coupling of SREHP to the CtxB protein did not significantly alter the immunogenicity of CtxB (123). These results confirmed the feasibility of generating mucosal immune responses to immunogenic peptides through their fusion to CtxB and established CtxB–SREHP-12 as one of the first candidates for an oral vaccine to prevent amebiasis.

An alternative approach was recently used by Beving et al., who orally immunized mice by simultaneous administration of the recombinant LC3 protein (which contains amino acids 758 to 1134 of the 170-kDa subunit of the Gal/GalNAc lectin fused to six histidine residues) and cholera toxin (6). Mice receiving this combination developed mucosal IgA anti-LC3 and anti-lectin antibody responses based on the analysis of intestinal
secretions derived from intestinal rinsing and homogenization of intestinal tissue. Importantly, the intestinal secretions derived from LC3-cholera toxin-immunized mice significantly inhibited the adherence of amebic trophozoites to mammalian cells in an in vitro assay, establishing that oral vaccination with a recombinant amebic antigen can induce adherence-blocking antibodies in mice.

The challenge now will be to demonstrate that both the CtxB–SREHP-12 fusion protein and some form of the 170-kDa subunit of the Gal/GalNAc lectin can be mucosally immunogenic in humans without the need for whole cholera toxin molecule as an adjuvant. One approach to this problem may come from work by Jobling and Holmes, who have developed constructs that allow for bacterial expression of an antigen such as MBP fused to the A2 (the CtxB-binding portion) segment of the cholera toxin A moiety (36). Expression of CtxB on the same plasmid allows the formation of “pseudotoxin” molecules, where assembly of the toxin molecule occurs but where the toxic CtxA domain is replaced by the antigen (in this example, MBP) of interest. We are currently testing a pseudotoxin where assembly of the toxin molecule occurs but where the toxic CtxA domain is replaced by the antigen (in this example, MBP) of interest.

FIG. 4. Model of the CtxA2-SREHP-MBP-CtxB and CtxA2-MBP-CtxB fusion proteins. The CtxA2-MBP-CtxB complex, engineered by Jobling and Holmes (36), contains the MBP fused to the A2 domain of cholera toxin. This protein can bind to live cholera toxin B subunits to create the MBP-pseudotoxin molecule shown on the right. In collaboration with Jobling and Holmes, I have created a new version of this protein, where a SREHP-MBP fusion protein is fused (at the carboxy terminus of SREHP) to the A2 domain of cholera toxin (96). This protein in turn can bind to pentameric cholera toxin B to create the SREHP-MBP-pseudotoxin molecule shown on the left.

Attenuated Salmonella spp. as Delivery Systems for the Recombinant SREHP Molecule

We have also explored the use of attenuated strains of Salmonella as carriers to deliver the SREHP antigen to induce mucosal and cell-mediated anti-amebic immune responses. Salmonella spp. are particularly attractive as vectors to deliver amebic antigens because oral vaccination with salmonellae expressing heterologous antigens can induce mucosal IgA, serum IgG, and cell-mediated immune responses to the foreign antigen (19). Since amebiasis begins as an intestinal infection, there is a clear rationale for attempting to induce a mucosal IgA response; however, once E. histolytica trophozoites invade through colonic mucosae, serum antibody or cell-mediated immune responses may become much more important in host defense. As noted above, protection from amebic liver abscess may be dependent on anti-amebic antibodies and cell-mediated immune responses; therefore, the wide range of host responses induced by salmonellae may be especially valuable in protection against amebiasis.

Curtiss and colleagues have developed strains of salmonellae that have engineered deletions in cyclic AMP receptor (crp) and adenyl cyclase (cya) genes, resulting in significant attenuation (18). In addition, the strains have an engineered deletion in the asd gene, which results in a requirement for diaminopimelic acid for bacterial growth (63). The asd gene deletion can be complemented by a plasmid copy, thus providing a non-antibiotic resistance-selectable marker for transformation which is extremely desirable in strains destined for human or animal vaccine use (63).

In initial studies, low-level expression of the SREHP-MBP fusion protein was achieved in Salmonella typhimurium Δ3987 (Δcya Δcrp Δasd), and this strain was used to orally vaccinate both mice and gerbils (13). While colonization of vaccinated animals with S. typhimurium expressing SREHP-MBP was demonstrated and the vaccine strain could be recovered from the spleens of vaccinated animals, indicating that the vaccine strain could reach lymphoid tissue, no anti-SREHP or anti-amebic antibodies were detected in vaccinated mice or gerbils (13). One potential explanation for this failure was simply inadequate levels of expression of the SREHP-MBP protein in these strains. Therefore, to improve the level of SREHP-MBP expression, a new SREHP-MBP plasmid with a pUC18 origin of replication was constructed that was capable of reaching high copy numbers in S. typhimurium (125). We found that S. typhimurium χ4550 (Δcya Δcrp Δasd) transformed with this plasmid (designated Stm-SREHP) produced high levels of the SREHP-MBP protein (15 to 20% of the total protein detected in SDS-PAGE-separated lysates of the vaccine strain). A three-dose oral vaccination of mice with Stm-SREHP induced mucosal IgA anti-amebic antibodies in the saliva and stool of vaccinated mice and IgG anti-amebic antibodies in the serum of vaccinated mice. Gerbils vaccinated with Stm-SREHP also developed mucosal IgA anti-amebic antibodies in stool and IgG anti-amebic antibodies in serum (125). Our ability to obtain mucosal and serum anti-amebic responses in animals after oral immunization with the new construct clearly indicated that, at least in the case of SREHP-MBP, the immunogenicity of a heterologous antigen delivered by S. typhimurium is directly dependent on the quantity of antigen produced by the vaccine carrier. Control mice and gerbils that were vaccinated with S. typhimurium χ4550 (Δcya Δcrp Δasd) containing the plasmid without the SREHP-MBP coding sequence (Stm-Ctrl) did not produce any detectable anti-amebic antibodies. Mice and gerbils vaccinated with Stm-SREHP or Stm-Ctrl developed mucosal and serum antibodies to S. typhimurium LPS, indicating that expression of the SREHP-MBP molecule did not alter the immunogenicity of the S. typhimurium carrier (125).

Because oral vaccination with Stm-SREHP had induced serum IgG responses to SREHP and amebae, we explored whether vaccination with Stm-SREHP protected gerbils against amebic liver abscess. Strikingly, of the gerbils vaccinated with Stm-SREHP, only 22% developed an amebic liver abscess after intrahepatic challenge with virulent E. histolytica trophozoites,
while 100% of Stm-Ctrl and nonvaccinated gerbils developed an amebic liver abscess after intrahepatic challenge (125).

These studies established that *S. typhimurium* could serve as an effective delivery system for the amebic SREHP molecule and demonstrated that vaccination by the oral route could provide protection against amebic liver abscess. A logical next step was to ask whether similar results could be obtained with expression of SREHP in attenuated strains of *S. typhi*, thus providing the possibility of a combination amebiasis-typhoid fever vaccine. The SREHP-MBP fusion protein has now been successfully expressed in *S. typhi* TY2 χ24397 (Δcrp Δarsd) (126). High-level expression of SREHP-MBP was achieved (10 to 15% of the total protein detected in Coomassie staining of SDS-PAGE-separated lysates), expression of the SREHP-MBP fusion protein did not alter the attenuation of the *S. typhi* vaccine strain based on its L50 in mice, and SREHP remained immunogenic, since mice parenterally immunized with *S. typhi* TY2 χ24397 (Δcrp Δarsd) expressing SREHP-MBP developed serum anti-amebic antibodies. While the inability of *S. typhi* to colonize mice by the oral route precluded studies of the mucosal immunogenicity of this construct, the data obtained suggest that the *S. typhi* TY2 χ24397 (Δcrp Δarsd) strain expressing SREHP-MBP represents a potential candidate for human trials.

**Limitations of Current Oral Vaccine Studies**

While the results of these recent studies are promising, some important caveats apply. The *S. typhi* TY2 χ24397 (Δcrp Δarsd) strain was studied in a previous clinical trial in humans and was found to be immunogenic (in terms of the induction of anti-lipopolysaccharide antibodies) and attenuated, but several individuals experienced fever and/or diarrhea with vaccination (105). A new *S. typhi* strain which includes a deletion mutation of the *cdt* gene (implicated in colonization of deep tissue), *S. typhi* TY2 χ4632 (Δcrp-ctd Δarsd), has recently been developed and has been used to express the pre-S1 protein of the hepatitis B virus (64). In a recent clinical trial in female adult volunteers, this strain retained immunogenicity (anti-lipopolysaccharide antibodies) when administered by the oral or rectal route and showed decreased reactogenicity. However, only one vaccinated individual showed seroconversion to the pre-S1 protein, a particularly disappointing result since, in a previous study, vaccination of mice with *S. typhimurium* expressing the same protein had resulted in seroconversion to pre-S1 protein in eight of nine orally vaccinated mice (89). These data emphasize that murine immune responses to heterologous antigens expressed in *S. typhimurium* may not predict the human immune response to the same antigen expressed in *S. typhi* and indicate that further modifications to the *S. typhi* (Δcrp-ctd Δarsd)-based system may be necessary before it can be used to reliably induce immune responses to heterologous antigens in humans.

Another unresolved problem is the aforementioned lack of a good animal model for intestinal amebiasis. This obviously affects the ability to study the efficacy of any of these oral vaccine constructs in preventing intestinal disease. How one selects the best oral vaccine candidates for testing in humans in the absence of efficacy testing in animal models remains a difficult question. Finally, even if these vaccines prove successful in stimulating a secretory IgA response to protective amebic antigens in humans, there are at present no data that allow us to predict the efficacy of such a response in protecting against amebiasis and the duration of any protective response that develops. In this regard, the report that *E. histolytica* can degrade human secretory IgA in vitro raises as yet unanswered questions about the efficacy of *E. histolytica*-specific secretory IgA in preventing amebic invasion in vivo (40).

**CONCLUSIONS**

The application of molecular biologic techniques to the study of amebiasis has produced a tremendous increase in our knowledge of the biology of *E. histolytica* and has enabled the rapid development of several candidate vaccines to prevent amebiasis. Studies with the gerbil model of amebic liver abscess have now identified three recombinant *E. histolytica* antigens that can protect animals from amebic liver abscess. Successful oral delivery of recombinant amebic antigens has been achieved, with the induction of mucosal immune responses in animals and successful protection of gerbils from amebic liver abscesses. However, no data are available on the efficacy of any of the candidate vaccines in preventing intestinal amebiasis. This is problematic, and in the absence of any reliable animal model for intestinal amebiasis, it may be impossible to adequately assess the protective efficacy of any candidate vaccine in preventing intestinal amebiasis before human trials can be performed. Despite these difficulties, there is reason for optimism. In addition to their value as potential vaccines to prevent amebiasis, constructs such as the CtxB-SREHP-12 protein are providing important information about the design of enteric vaccines and new insights into the requirements for mucosal immunogenicity. The recent demonstration that the recombinant amebic SREHP antigen can be mucosally immunogenic when delivered by attenuated *V. cholerae* or *S. typhi* strains provides additional evidence for the feasibility of combination vaccines, designed to be effective against multiple enteric pathogens. This is an especially welcome development, given the profound limitations in health care resources in many regions where enteric infections are endemic. Given these financial constraints, “bundling” an *E. histolytica* vaccine into a combination enteric vaccine may represent the most cost-effective method of delivering protection against amebiasis into the regions of the world where the vaccine is needed most. The first steps in the process of developing an oral combination enteric vaccine that could prevent amebiasis have now been achieved, but the major question of the safety and efficacy of these candidate vaccines in humans remains unanswered.

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