Oral Immunization with Attenuated Vaccine Strains of *Vibrio cholerae* Expressing a Dodecapeptide Repeat of the Serine-Rich *Entamoeba histolytica* Protein Fused to the Cholera Toxin B Subunit Induces Systemic and Mucosal Antiamebic and Anti-*V. cholerae* Antibody Responses in Mice

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Entamoeba histolytica is a significant cause of morbidity and mortality worldwide. The serine-rich *E. histolytica* protein (SREHP) is a surface-expressed trophozoite protein that includes multiple hydrophilic tandem repeats. A purified fusion protein between the dodecapeptide repeat of SREHP and cholera toxin B subunit (CTB) has previously been shown to be immunogenic in mice after oral inoculation when cholera toxin is coadministered as an immunoadjuvant. We engineered a live attenuated El Tor *Vibrio cholerae* vaccine strain, Peru2, to express the SREHP-12-CTB fusion protein to the supernatant from either a plasmid [Peru2 (pETR5.1)] or from a chromosomal insertion (ETR3). Vector strains were administered orally to germfree mice that were subsequently housed under nongermfree conditions; mice received one (day 0) or two (days 0 and 14) inoculations. No immunoadjuvant or cholera holotoxin was administered. Mice that received two inoculations of Peru2(pETR5.1) had the most pronounced antiamebic systemic and mucosal immunologic responses. Less marked, but significant, anti-SREHP serum immunoglobulin G antibody responses were also induced in mice that received either one or two oral inoculations of strain ETR3. Anti-*V. cholerae* responses were also induced, as measured by the induction of serum vibriocidal antibodies and by serum and mucosal anti-CTB antibody responses. These results suggest that *V. cholerae* vector strains can be successful delivery vehicles for the SREHP-12-CTB fusion protein, to induce mucosal and systemic antiamebic and anti-*V. cholerae* immune responses. The magnitude of these responses is proportional to the amount of SREHP-12-CTB produced by the vector strain.

Entamoeba histolytica is an intestinal protozoan parasite of humans that causes significant morbidity and mortality worldwide (24). The invasion of the colonic surface by *E. histolytica* trophozoites can cause severe colitis. Hematogenous dissemination of trophozoites can cause extraintestinal, usually hepatic, abscess formation. The attachment of trophozoites to intestinal epithelial cells appears to be a crucial first step in initiating this pathophysiology (15, 19, 25). Parenteral vaccination with a number of surface-expressed *E. histolytica* antigens has shown that immune responses protective against invasive hepatic amebiasis can be induced in a standard gerbil animal model (30–32, 40, 42). The serine-rich *E. histolytica* protein (SREHP) is one such antigen that has been shown to induce protective immunity (39–43). SREHP is a surface-expressed amebic glycoprotein that contains multiple hydrophilic dodecapeptide and octapeptide tandem repeating subunits; its function remains unclear (33, 34, 40). Parenteral vaccination of gerbils with an approximately 200-amino-acid SREHP moiety fused to maltose-binding protein (SREHP-MBP) resulted in protection of 64 to 100% of animals after direct hepatic inoculation with virulent amebic trophozoites (40, 42). In addition, passive administration of anti-SREHP antibodies prevented

amebic liver abscess formation in a severe combined immunodeficiency mouse model of amebiasis and was correlated with the ability of antiserum to block *E. histolytica* adhesion (39).

Although induction of systemic immune responses may be crucial in preventing or limiting extraintestinal manifestations of amebiasis (29), the induction of protective mucosal immune responses at the intestinal surface could abrogate trophozoite attachment and possibly limit or prevent intestinal and extraintestinal amebiasis (25). Expression of heterologous antigens by attenuated strains of bacteria at mucosal surfaces is one method of inducing potent mucosal immune responses against heterologous antigens. Indeed, protective immune responses against amebiasis have been induced in gerbils after oral inoculation with an attenuated strain of *Salmonella typhimurium* expressing the SREHP-MBP fusion protein. This immunologic induction was directly correlated with the level of expression of fusion protein by vector strains (10, 43).

The use of live attenuated strains of *Vibrio cholerae* to deliver heterologous antigens to mucosal surfaces has many advantages over other vector systems. *V. cholerae* is noninvasive but still elicits prominent and long-lasting systemic and mucosal immune responses (13, 17, 23). Well-studied attenuated strains which previously have been used successfully to deliver heterologous antigens for induction of immunological responses at mucosal surfaces have been developed (5, 6, 16, 18, 20, 22, 35, 38). Certain heterologous antigens can be fused to the nontoxic B subunit of cholera toxin (CTB) and can thus be

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype ^a	Source or reference
<i>V. cholerae</i>		
C6709	El Tor, Inaba, wild type; Sm ^r	5, 26
Peru2	C6709 $\Delta attRS1$; Sm ^r	5, 26
ETR3	Peru2 $\Delta lacZ lacZ::lppP$, $lacPO \rightarrow ompA2::SREHP-12::ctxB$; Sm ^r	This study
<i>E. coli</i> JM105	<i>thi rpsL endA sbcB15 hsdR4 supE</i> $\Delta(lac-proAB)$ F'[<i>traD36 proAB⁺ lacI^q lacZ</i> Δ M15]; Sm ^r	Pharmacia P-L Biochemicals Inc., Milwaukee, Wis.
Plasmids		
pKK223-3	pBR322-based plasmid containing a multiple cloning site between <i>tacP</i> and the <i>rnnB</i> transcriptional terminator; Amp ^r	Pharmacia P-L Biochemicals Inc., Milwaukee, Wis.
pMCSETR1B	pKK223-3 with the <i>tac</i> promoter replaced by a fragment containing additional restriction sites (<i>Bam</i> HI- <i>Nsi</i> I- <i>Xba</i> I- <i>Eco</i> RV- <i>Bgl</i> II- <i>Spe</i> I- <i>Eco</i> RI- <i>Pst</i> I); Amp ^r	This study
pVA-II	pINIII <i>ompA2</i> plasmid, with <i>lppP</i> , $lacPO \rightarrow ompA2::SREHP-12::ctxB$; Amp ^r	41
pETR5	pMCSETR1B with an 840-bp <i>Bgl</i> II- <i>Pst</i> I fragment amplified from pVA-II, encoding <i>lppP</i> , $lacPO \rightarrow ompA2::SREHP-12::ctxB$ cloned between <i>Bgl</i> II and <i>Pst</i> I sites; Amp ^r	This study
p6891MCS	pBR327-based plasmid containing approximately 8 kbp of <i>V. cholerae lacZ</i> with a multiple cloning site inserted into the <i>Kpn</i> I site; Amp ^r	5
pETR5.1	1.8-kbp <i>Bgl</i> II- <i>Pvu</i> I fragment of pETR5, including <i>lppP</i> , $lacPO \rightarrow ompA2::SREHP-12::ctxB$ and the <i>rnnB</i> transcriptional terminator, made blunt ended and ligated into the <i>Stu</i> I site of p6891MCS; Amp ^r	This study

^a Amp^r, ampicillin resistant; Sm^r, streptomycin resistant.

secreted to the extracellular milieu by *V. cholerae* and taken up by intestinal microfold (M) cells (2, 3, 11, 13, 21, 28).

Vaccination of mice with a recombinant fusion protein of a dodecapeptide, derived from a repeating subunit of SREHP, fused to the amino terminus of CTB (SREHP-12-CTB) has previously been shown to induce both mucosal and systemic anti-SREHP and anti-CTB immune responses when the fusion protein was administered orally with subclinical doses of cholera holotoxin as an immunoadjuvant (41).

In this study, we wished to examine whether SREHP-12-CTB could be expressed in vivo, by a live oral attenuated *V. cholerae* vector, to stimulate an immune response comparable to administration of the purified protein with immunoadjuvant. We utilized an attenuated El Tor *V. cholerae* vaccine strain (Peru2) and expressed SREHP-12-CTB from both chromosomal and plasmid constructs. We made use of a recently described germfree mouse model of *V. cholerae* infection to assess antiamebic and anti-*V. cholerae* mucosal and systemic humoral immune responses (8). We found that attenuated strains of *V. cholerae* can act as successful delivery vehicles for induction of antiamebic and anti-*V. cholerae* mucosal and systemic immune responses even without cholera holotoxin and that successful induction of an antiamebic response was directly related to the level of expression of the SREHP-12-CTB fusion protein from the vaccine constructs.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in this study are detailed in Table 1. All strains were maintained at -70°C in Luria-Bertani (LB) medium (27) containing 15% glycerol. LB medium contained ampicillin (100 $\mu\text{g}/\text{ml}$), streptomycin (100 $\mu\text{g}/\text{ml}$), or X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; 80 $\mu\text{g}/\text{ml}$; International Biotechnology Inc., New Haven, Conn.) as appropriate. All cultures were grown at 37°C with aeration.

Genetic methods. Isolation of plasmid and bacterial chromosomal DNA, restriction enzyme digestion, agarose gel electrophoresis, and Southern hybridization of DNA separated by electrophoresis were performed by standard biological techniques (27). GeneScreen Plus hybridization transfer membranes (DuPont Biotechnology Systems, NEN Research Products, Boston, Mass.) were used as described in the manufacturer's protocol for Southern hybridization. DNA sequencing was performed at the DNA Sequencing Core Facility, Department of Molecular Biology, Massachusetts General Hospital, by use of ABI Prism DiTerminator cycle sequencing with AmpliTaq DNA polymerase FS with an ABI 377 DNA Sequencer (Perkin-Elmer Applied Biosystems Division, Foster City, Calif.).

Plasmids were transformed into *Escherichia coli* JM105 by standard techniques

or were electroporated into *V. cholerae*, with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.), as instructed by the manufacturer, and modified for electroporation into *V. cholerae* as described previously (12). Electroporation conditions were 2,500 V at 25- μF capacitance, producing time constants of 4.6 to 4.8 ms.

DNA restriction endonucleases, T4 DNA ligase, the Klenow fragment of DNA polymerase I, and calf intestinal alkaline phosphatase were used as described in the manufacturers' specifications. Restriction enzyme-digested chromosomal and plasmid DNA fragments were separated on 1% agarose gels; the required fragments were cut from the gel under UV illumination and recovered by the freeze-squeeze technique (36). Vent DNA polymerase thermocyclic reaction products were prepared in volumes of 100 μl containing 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 20 mM Tris-HCl (pH 8.8 at 24°C), 0.1% Triton X-100 (1 $\times$ ThermoPol reaction buffer), 200 μM each deoxynucleoside triphosphate, 0.25 μM each primer, 4 mM MgSO_4 , and 1 U of Vent DNA polymerase (New England Biolabs, Beverly, Mass.). The temperature cycling for the amplification reaction was performed in a PTC-100 programmable thermal controller thermocycler (MJ Research Inc., Watertown, Mass.) as follows: 94°C for 5 min, 25 cycles of 94°C for 1 min (denaturing), 50°C for 1 min (annealing), 72°C for 1 min (extension), and 72°C for 5 min (terminal extension).

Genetic constructions. Plasmid pVA-II has been described previously (41) and contains the dodecapeptide repeat of SREHP upstream and in frame with the tyrosine residue of the CTB leader sequence and downstream and in frame with the *OmpA2* leader peptide, with the fusion under the transcriptional control of tandemly arranged *E. coli lpp* and *lac* promoters. Plasmid pETR5 was constructed by cloning an 840-bp fragment containing *lppP*, $lacPO \rightarrow ompA2::SREHP-12::ctxB$, amplified by PCR from pVA-II and incorporating *Bgl*II and *Pst*I ends, into the unique *Bgl*II and *Pst*I sites of pMCSETR1B to provide a downstream transcriptional terminator. The ligation product was transformed into *E. coli* JM105, and pETR5 was confirmed by restriction enzyme digestion and sequence analysis. A 1.8-kbp *Bgl*II-*Pvu*I fragment of pETR5 containing *lppP*, $lacPO \rightarrow ompA2::SREHP-12::ctxB$, and the transcriptional terminator *rnnB* was blunt ended by treatment with the Klenow fragment of DNA polymerase I and ligated into the *Stu*I site of p6891MCS. The ligation product was transformed into *E. coli* JM105, and the plasmid product, pETR5.1, containing *lppP*, $lacPO \rightarrow ompA2::SREHP-12::ctxB$, and *rnnB* within an 8-kbp fragment of *V. cholerae lacZ*, was confirmed by restriction digestion. Plasmid pETR5.1 was electroporated into *V. cholerae* Peru2, a *V. cholerae* El Tor Inaba strain deleted for *attRS1* sequences and the entire CTX genetic element (5, 26, 37). The fragment containing *lppP*, $lacPO \rightarrow ompA2::SREHP-12::ctxB$, and *rnnB* was inserted into the chromosomal copy of *lacZ* by in vivo marker exchange, as described previously (5), to create strain ETR3. Strains undergoing marker exchange between pETR5.1 and the chromosomal copy of *lacZ* were white on X-Gal media and ampicillin susceptible. The correct chromosomal insertion was confirmed by Southern hybridization (data not shown).

Characterization of SREHP-12-CTB. The supernatant of an overnight culture of Peru2(pETR5.1) was concentrated in Centrifuil 3 concentrators (Amicon Inc., Beverly, Mass.) and run on a Tris-Tricine sodium dodecyl sulfate-polyacrylamide gradient (4 to 15%) gel in a Mini-Protein II gel apparatus (Bio-Rad Laboratories, Hercules, Calif.) as described in the manufacturer's specifications and then stained with Coomassie blue. For Western blotting (immunoblotting), separated proteins were transferred to Hybond enhanced chemiluminescence

nitrocellulose membranes (Amersham Life Sciences, Buckinghamshire, England) with a semidry blotting apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). Immunoreactive proteins were visualized after sequential incubation with polyclonal rabbit anti-cholera toxin antiserum (1:1,000; Sigma Chemical Co., St. Louis, Mo.) and goat anti-rabbit immunoglobulin G (IgG)-conjugated alkaline phosphatase (1:2,000; Sigma) and then stained for phosphatase activity as described previously (14). Comparison was made to purified CTB (0.5 µg; Sigma).

Whole-cell extracts were prepared by freeze-thawing and sonication, and periplasmic extracts were prepared as described previously (14). SREHP-12-CTB was measured in cell fractions or unconcentrated supernatants by a quantitative enzyme-linked immunosorbent assay (ELISA) for CTB. Serial dilutions of samples in phosphate-buffered saline–0.05% Tween 20 (PBS-T; undiluted to 1:2,187) were applied to 96-well microtiter plates previously coated with 100 ng of type III ganglioside (Sigma) per well in 50 mM carbonate buffer (pH 9.6) and previously blocked with PBS–1% bovine serum albumin (Sigma; PBS-BSA). Plates were incubated at 37°C for 1 h and then washed with PBS-T. A 1:2,000 dilution of goat anti-CTB (List Biological Laboratories, Inc., Campbell, Calif.) in PBS-T was applied to each well, and the plates were incubated at 37°C for 1 h. After the wells were washed in PBS-T, a 1:4,000 rabbit anti-goat IgG–horseradish peroxidase conjugate (Southern Biotechnology Associates Inc., Birmingham, Ala.) was applied to each well; after incubation at 37°C for 1 h, the plates were developed with a 1-mg/ml solution of 2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) (ABTS; Sigma) with 0.1% H₂O₂ (Sigma) and the optical density at 405 nm was read in a Vmax microplate reader (Molecular Devices Corp., Sunnyvale, Calif.). Measured optical densities were compared to a standard curve provided by dilutions of purified CTB (List) in PBS-T.

SREHP-12-CTB was also analyzed by a modification of this ELISA employing ganglioside binding for capture but detection with a 1:200 dilution of mouse ascites containing the anti-SREHP-12 monoclonal antibody 2D4 (41, 43) in PBS-T, followed by incubation with a 1:2,000 goat anti-mouse IgG–horseradish peroxidase conjugate (Southern Biotechnology Associates) and detection as described above.

Inoculation and colonization of germfree mice. The development of an animal model for *V. cholerae* infection, by employing the oral inoculation of germfree mice that are subsequently housed in nongermfree conditions, has been described recently (8). Immediately upon removal of mice from their germfree shipper, five groups of five to six germfree female Swiss mice, 3 to 4 weeks old, (Taconic Farms, Inc., Germantown, N.Y.) were orally inoculated with approximately 10⁹ organisms of various *V. cholerae* strains resuspended in 0.5 M NaHCO₃ (pH 8.0) via gastric intubation. The mice were subsequently housed in nongermfree conditions. Two additional mice were removed from their germfree shipper, left uninoculated, and also moved to nongermfree conditions to act as unvaccinated control animals. Three groups of five to six mice each received a second oral inoculation on day 14. To assess colonization, fresh stool samples were collected immediately upon passage from all mice every 48 h throughout the study, resuspended in 500 µl of LB medium, vortexed, and allowed to settle. One-hundred-microliter aliquots were plated on thiosulfate-citrate-bile salts-sucrose medium, as well as LB medium containing either streptomycin or both streptomycin and ampicillin, to assess intestinal passage of the *V. cholerae* strains of interest.

Immunological sampling. Mice were sacrificed on day 28, at which time blood was collected via retroorbital and intracardiac puncture. Blood was allowed to clot, and serum was separated by centrifugation. Bile (3 to 6 µl) was collected via hepatic dissection and subsequent aspiration of the gallbladder (4). Fresh stool pellets were also collected for immunological evaluation. Each pellet was placed in 1 ml of a 3:1 mixture of PBS–0.1 M EDTA containing soybean trypsin inhibitor (type II-S; Sigma) at a concentration of 0.1 mg/ml and then vortexed until the pellets were broken. The mixture was centrifuged at 270 × g for 10 min, and the recovered supernatant was centrifuged at 17,000 × g for 10 min. The supernatant was recovered, and 20 µl of 100 mM phenylmethylsulfonyl fluoride (Sigma) was added to each 1 ml of recovered supernatant (41). Stool, biliary, and serum samples were aliquoted and stored at –70°C for subsequent analysis.

Detection of vibriocidal antibodies. Serum vibriocidal antibody titers were measured in a microassay as follows. The endogenous complement activity of test sera was inactivated by heating the sera to 56°C for 1 h. Fifty-microliter aliquots of serial twofold dilutions of test sera in PBS (1:25 to 1:25,600) were placed in the wells of sterile 96-well tissue culture plates; 50 µl of a 10⁸-CFU/ml culture of *V. cholerae* Peru2 in PBS with 22% guinea pig complement (Gibco BRL Life Technologies, Gaithersburg, Md.) was added to the serum dilutions, and the mixtures were incubated for 1 h at 37°C. One hundred fifty microliters of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) was added to each well, and the plates were incubated for approximately 2 h at 37°C. The optical density at 600 nm was then measured; the vibriocidal titer was calculated as the dilution of serum causing 50% reduction in optical density compared with that of wells containing no serum (5, 8).

Measurement of anti-CTB and anti-SREHP systemic and mucosal antibody responses. To detect anti-CTB antibody responses, microtiter plates were coated with 100 ng of type III gangliosides (Sigma) per well in 50 mM sodium carbonate buffer (pH 9.6). Following overnight incubation at room temperature and three washes in PBS-T of each plate, 100 ng of CTB in carbonate buffer was applied to each well and the plates were again incubated overnight at room temperature. Following washing in PBS-T, the plates were blocked with PBS-BSA (1%).

To detect anti-CTB IgG antibody in sera, duplicate samples of serial dilutions

FIG. 1. Western blot of supernatants from Peru2 (lane 1), Peru2(pETR5.1) (lane 2), and purified CTB (0.5 µg) (lane 3), developed with antisera to CTB. The numbers on the right indicate the approximate positions of molecular size markers (in kilodaltons).

(1:160 to 1:81,920) of sera were placed in wells of microtiter plates previously coated with ganglioside-CTB and blocked with PBS-BSA. The plates were incubated at room temperature overnight and washed in PBS-T. A 1:2,000 dilution in PBS-T of goat anti-mouse IgG conjugated to biotin (Kirkegaard & Perry Laboratories [KPL], Gaithersburg, Md.) was applied to each well, and the plates were incubated overnight at room temperature. After the plates were washed in PBS-T, a 1:4,000 dilution of streptavidin-horseradish peroxidase conjugate (Zymed Laboratories Inc., South San Francisco, Calif.) was added to each well and the plates were incubated at room temperature for 2 h. Following washing in PBS-T, the plates were developed for peroxidase activity as described above and the optical density at 405 nm was measured. End dilutions were calculated as the highest dilution of test sample producing an optical density greater than three times the mean of 16 control wells to which PBS-T without serum was added.

To detect anti-CTB IgA responses in sera, bile, and stool, duplicate serial twofold dilutions of serum (1:12.5 to 1:6,400), bile (1:150 to 1:76,800), and stool (1:1 to 1:512) samples in PBS-T were added to microtiter wells. Anti-CTB IgA responses were assayed by use of a 1:2,000 goat anti-mouse IgA–biotin conjugate (KPL) as the detection antibody. To measure the total amount of IgA in each stool preparation, 96-well ELISA plates were coated with 0.1 ml of 1:5 dilutions of stool aliquots. The plates were blocked with PBS-BSA, and total IgA was detected with a 1:2,000 dilution of biotin-labelled goat anti-mouse IgA (KPL) followed by streptavidin-horseradish peroxidase (Zymed), with development done as described above. Comparisons were made to a mouse IgA standard (Kappa TEPC 15; Sigma).

To detect anti-SREHP-12 IgG antibodies in day 28 sera and anti-SREHP-12 IgA responses in sera, bile, and stool, the ELISA protocols described above, using plates coated with *E. histolytica* HM1:IMSS trophozoites in PBS (10⁴ per well) overnight at 4°C and blocked in PBS-BSA as described previously, were followed (41, 43).

Statistics and graphics. Data were plotted by use of CA-Cricket Graph Software (Computer Associates, Garden City, N.Y.), and statistical significance was analyzed by the two-tailed *t* test for the comparison of means.

RESULTS

Construction of vector strains. Oral, live attenuated vaccine vectors of *V. cholerae* were derived from Peru2, a Peruvian El Tor strain deleted for *attRS1* and the entire CTX genetic element (26, 37). A genetic fusion of *ompA2::SREHP-12::ctxB* and *rrnB* was placed under the transcriptional control of the tandemly arranged *E. coli lpp* and *lac* promoters within the *V. cholerae lacZ* gene on plasmid pMCS6891 to create plasmid pETR5.1. After introduction of pETR5.1 into Peru2, the construct was maintained in plasmid form by maintaining ampicillin selection pressure [Peru2(pETR5.1)]. The fragment containing *lppP*, *lacPO*→*ompA2::SREHP-12::ctxB*, and *rrnB* was also introduced within the chromosomal copy of the Peru2 *lacZ* gene by marker exchange to create vaccine strain ETR3. The *lac* promoter is expressed in *V. cholerae* without any induction because this species lacks a functional *lacI* gene.

Expression of SREHP-12-CTB by *V. cholerae* vector strains in vitro. To ascertain whether the SREHP-12-CTB fusion protein was secreted to the supernatant by *V. cholerae*, supernatant fractions were separated by gel electrophoresis; the gels were stained with Coomassie blue (data not shown) or developed by Western blotting with antiserum directed against CTB

FIG. 2. Results of ELISA of supernatant and control samples, with GM₁ ganglioside as the capture molecule and mouse monoclonal anti-SREHP 2D4 antibody used for detection. The supernatant from Peru2(pETR5.1) gave a significantly higher reading than the other samples did. *, $P < 0.05$. OD, optical density.

(Fig. 1). The SREHP-12-CTB fusion protein was found to migrate with a molecular mass of approximately 15 kDa as reported previously (41), slightly larger than native CTB, as expected. To verify that SREHP-12 and CTB remained fused after secretion by *V. cholerae*, an ELISA was performed on supernatant fractions using GM₁ ganglioside as the capture molecule and mouse ascites containing the anti-SREHP-12 monoclonal antibody 2D4 as the detection antibody (41, 43). As shown in Fig. 2, supernatant fractions of Peru2(pETR5.1) contained a molecule that was bound by GM₁ ganglioside and detected by the anti-SREHP-12 monoclonal antibody, whereas the monoclonal antibody did not detect 200 μ g of CTB per ml itself, immunoreactive material in the supernatant of Peru2, or supernatant of Peru2 with 200 μ g of CTB per ml added.

The amount of SREHP-12-CTB secreted to the supernatant

by the vaccine strains was quantitated by an ELISA technique standardized to the CTB component. Unconcentrated supernatant samples of ETR3 contained approximately 10 ng of SREHP-12-CTB per ml per optical density unit at 600 nm of overnight culture. Unconcentrated supernatant samples of Peru2(pETR5.1) grown in the presence of ampicillin contained approximately 1 to 10 μ g/ml/optical density unit at 600 nm, approximately 100- to 1,000-fold more than that expressed from the single copy of the gene in ETR3.

Cellular localization studies performed by quantitative ELISA disclosed that all of the SREHP-12-CTB produced by ETR3 was localized to the supernatant (Fig. 3A). In a culture of Peru2(pETR5.1), approximately 82% was localized to the supernatant, 16% was localized to the periplasm, and 2% was localized to the cytosolic fraction (Fig. 3B).

Intestinal colonization of mice with vector strains. Recent reports have suggested that heterologous antigens expressed from plasmid constructs in *V. cholerae* can result in immune responses even without ongoing antibiotic selective pressure or the use of balanced lethal mutations to maintain the plasmid (1, 7). To ascertain the length of intestinal passage of *V. cholerae* vector strains and to measure the duration of passage of organisms containing plasmid pETR5.1, freshly passed mouse stools were analyzed on selective media (Fig. 4). After the first oral inoculation, mice continued to pass *V. cholerae* in their stool for 7 to 14 days. After the second inoculation, mice cleared *V. cholerae* from their stools more rapidly, within 2 days. Among those mice that received Peru2(pETR5.1), *V. cholerae* containing the plasmid was present in stool for 3 to 5 days after the first inoculation and for 1 day after the second inoculation. Mice that received Peru2(pETR5.1) continued to pass the *V. cholerae* vector in a similar fashion to mice that received Peru2 or ETR3, despite the loss of the plasmid.

Measurement of serum vibriocidal antibodies. To ascertain the ability of the mice to mount an immune response to the vector construct used for delivery of the heterologous antigen, vibriocidal antibodies directed against the bacteria itself were measured. Responses were compared with those of control mice (uninoculated germfree mice that were housed identically). On day 28, mice that had received a single inoculation of Peru2 or ETR3 on day 0 had low titers of vibriocidal antibody (Fig. 5). Mice that had received an additional inoculation on

FIG. 3. Percentage of total SREHP-12-CTB localized to various cellular compartments. (A) Strain ETR3; (B) strain Peru2(pETR5.1).

FIG. 4. Aggregate percentages of mice (solid bars) with *V. cholerae* recovered from stool cultures following oral inoculations on days 0 and 14 with Peru2, ETR3, and Peru2(pETR5.1). The percentages of mice receiving Peru2 (pETR5.1), in which the plasmid was still present in *V. cholerae* vector strains recovered from stool following inoculation on day 0 and day 14, are also represented (hatched bars). Recovery of the vector strain itself following inoculation with Peru2(pETR5.1) did not differ from recovery following inoculation with Peru2 or ETR3.

day 14 had increased vibriocidal antibody levels consistent with a booster effect.

Immunogenicity of the SREHP-12-CTB fusion protein. Day 28 immunological responses observed in mice that received a single inoculation of ETR3 were compared to those observed in mice that received a single inoculation of the control strain Peru2. Similarly, immunological responses in mice that received two inoculations of vector strains ETR3 or Peru2 (pETR5.1) were compared to those observed in mice that received two inoculations of the control strain Peru2.

As shown in Fig. 6A, the most pronounced anti-CTB IgG serum antibody response was observed in mice that received two inoculations of Peru2(pETR5.1) ($P < 0.001$). Mice that received two inoculations of ETR3 also had a significant response ($P < 0.001$). The highest anti-SREHP IgG serum antibody response was observed in mice that received two inoculations of Peru2(pETR5.1) ($P < 0.01$) (Fig. 7A). Mice inoculated with the vaccine strain ETR3 also developed significant serum anti-SREHP IgG antibody responses on day 28 after single ($P < 0.02$) or double ($P < 0.001$) oral inoculations (Fig. 7A). Serum from day 28 analyzed for anti-SREHP IgA response was not significantly different from that of control animals (data not shown); however, a significant anti-CTB IgA response was detected in sera from day 28 from mice that received two inoculations of Peru2(pETR5.1) ($P < 0.001$) (Fig. 6B).

Stool preparations from all mice were found to contain equivalent amounts of total IgA (data not shown). The most prominent IgA anti-CTB response in stool was observed in mice that received two inoculations of Peru2(pETR5.1) ($P < 0.001$), although significant but smaller IgA responses were observed in mice that received one ($P < 0.01$) or two inoculations of ETR3 ($P < 0.01$) (Fig. 6C). Similarly, the most prominent anti-SREHP IgA response in stool was observed in mice that received two inoculations of Peru2(pETR5.1) ($P < 0.01$) (Fig. 7B).

The most prominent IgA anti-CTB response in bile collected

on day 28 was observed in mice that received two inoculations of Peru2(pETR5.1) ($P < 0.001$) or two inoculations of ETR3 ($P < 0.01$) (Fig. 6D). A biliary IgA anti-SREHP was observed only in mice that received two inoculations with Peru2 (pETR5.1) ($P < 0.01$) (Fig. 7C).

DISCUSSION

A number of in vitro studies suggest that *E. histolytica* trophozoite-target cell contact is required for ameba-mediated cytolysis of mammalian cells (15, 19, 25). Salivary secretory IgA from patients previously infected with *E. histolytica* has been shown to inhibit amebic adhesion to mammalian cells (9), and the ability of antisera to protect against amebic liver abscess formation in the severe combined immunodeficiency mouse model of amebiasis is associated with the ability of antisera to block *E. histolytica* adhesion (39). Antibodies directed against a surface-expressed *E. histolytica* trophozoite protein, SREHP, have been shown to block amebic adherence (33, 34, 39). A SREHP-12-CTB fusion protein has recently been shown to induce mucosal and systemic immune responses after oral inoculation in mice when subclinical doses of cholera holotoxin are coadministered as an immunoadjuvant. In an attempt to develop a safe, effective vaccine that induces immune responses against *E. histolytica*, we examined the ability of live, attenuated vector strains of *V. cholerae* expressing SREHP-12-CTB to induce antiamebic and anti-*V. cholerae* mucosal and systemic immune responses.

We were able to develop *V. cholerae* vector constructs that expressed the SREHP-12-CTB fusion protein from a stable chromosomal insertion or from a plasmid construct. We found that *V. cholerae* vector strains secreted SREHP-12-CTB to the supernatant, that SREHP-12 and CTB moieties remained attached during the secretory process, and that the amount of fusion protein produced was highest in strains expressing SREHP-12-CTB from a plasmid construct. We found that *V. cholerae* vector strains expressing SREHP-12-CTB effectively colonized mice after oral inoculation and that even in the absence of ongoing plasmid selection pressure, mice continued to pass strains of *V. cholerae* containing plasmid constructs for

FIG. 5. Geometric mean titer (GMT) of vibriocidal antibody responses on day 28, following no, one (day 0), or two (days 0 and 14) oral inoculations with various strains. Bars depict the standard errors of the mean for each group.

FIG. 6. Anti-CTB ELISA results on day 28 for mice inoculated with Peru2, ETR3, or Peru2(pETR5.1). "(S)" following the strain designation indicates groups of mice that received a single inoculation (day 0). "(D)" following the strain designation indicates groups of mice that received two inoculations (days 0 and 14). Results are reported as the end geometric mean titers (GMTs). Bars depict standard errors of the mean for each group. + and #, $P < 0.01$ and $P < 0.001$, respectively, compared with control group (Peru2; single or double dose). (A) Serum IgG; (B) serum IgA; (C) stool IgA; (D) bile IgA.

3 to 5 days in stool after oral inoculation. We found that the ability to induce an immunological response was correlated with the amount of SREHP-12-CTB being produced, with the most prominent anti-SREHP-12 and anti-CTB immune responses being observed in mice that received the *V. cholerae* vector strain containing the plasmid construct [Peru2(pETR5.1)]. We were able to demonstrate significant antiamebic (anti-SREHP) and anti-*V. cholerae* (anti-CTB) systemic and mucosal immunologic responses and the induction of vibriocidal antibody responses. The successful induction of both mucosal and systemic anti-SREHP-12 immune responses by this vector system could provide for two levels of protection against infection with *E. histolytica*. Mucosal anti-SREHP-12 immune responses could inhibit attachment of *E. histolytica* trophozoites to intestinal epithelial cells, and systemic immunity could

limit pathogenicity should invasive intestinal and extraintestinal amebiasis still occur.

In summary, we have shown that *V. cholerae* vaccine vector strains can act as successful delivery vehicles for inducing systemic and mucosal immune responses against SREHP-12-CTB even when no concomitant immunoadjuvant is administered. Such a vaccine system may be able to provide dual protection against the action of two important enteric pathogens, *V. cholerae* and *E. histolytica*. We have also shown that the newly developed mouse model of *V. cholerae* infection can be used to judge the ability to elicit an immune response to heterologous antigens produced by *V. cholerae* vector strains and that plasmid constructs can be used to induce immune responses in mice to heterologous antigens expressed by *V. cholerae* vector strains.

FIG. 7. Anti-SREHP ELISA results at day 28. "(S)" following the strain designation indicates groups of mice that received a single inoculation (day 0). "(D)" following the strain designation indicates groups of mice that received two inoculations (days 0 and 14). Results are reported as the end geometric mean titers (GMTs) for each group of mice. Bars depict standard errors of the mean for each group. **, +, and #, $P < 0.02$, $P < 0.01$, and $P < 0.001$, respectively, compared with control group (Peru2; for single or double dose) (A) Serum IgG; (B) stool IgA; (C) bile IgA.

To increase the amount of heterologous antigen produced by *V. cholerae* vector strains, we are evaluating antigenic expression from a number of high-level in vivo-activated promoters in *V. cholerae* vector strains. The development of a stable balanced lethal mutation system to maintain plasmids in *V. cholerae* could also be employed. The possibility of boosting the immunological response with immunoadjuvants and the evaluation of additional immunogenic antigens from *E. histolytica* are also being examined. To judge the ability to induce protective immunity against invasive amebiasis with vaccine constructs employing *V. cholerae* as the delivery vehicle, new animal models or adaptations of current animal models will need to be pursued, to allow assessment of protective efficacy following challenge.

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