Coexpression of the B Subunit of Shiga Toxin 1 and EaeA from Enterohemorrhagic *Escherichia coli* in *Vibrio cholerae* Vaccine Strains

JOAN R. BUTTERTON,^{1*} EDWARD T. RYAN,¹ DAVID W. K. ACHESON,²
AND STEPHEN B. CALDERWOOD^{1,3}

Infectious Disease Unit, Massachusetts General Hospital, Boston, Massachusetts 02114¹; Division of Geographic Medicine and Infectious Diseases, New England Medical Center, Boston, Massachusetts 02112²; and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115³

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A promoterless gene for the Shiga toxin 1 B subunit (*stxB*₁) has been placed under transcriptional control of the *Vibrio cholerae* heat shock gene *hspG*. A chromosomal enterohemorrhagic *Escherichia coli* fragment containing *eaeA* and 400 bp of upstream DNA was added to the construct, downstream of *stxB*₁; no transcription terminators were located between the two genes. The plasmid construct was confirmed by DNA sequencing; in vitro transcription-translation studies demonstrated expression of EaeA from the plasmid. The *hspG*→*stxB*₁, *eaeA* construct was inserted into *lacZ* on the chromosome of Peru2, an El Tor *V. cholerae* strain with both *attRS1* sequences and the entire cholera toxin genetic element deleted, and into *lacZ* in JRB10, a Peru2 derivative that has a second copy of *hspG*→*stxB*₁ also inserted in the *V. cholerae* virulence gene *irgA*. Two plasmid constructs, one containing *stxB*₁ under the control of the *tac* promoter and another containing *hspG*→*stxB*₁,*eaeA*, were transformed into Peru2. Expression of StxB1 by these constructs was quantified by enzyme-linked immunosorbent assay and was highest in the plasmid construct with *stxB*₁ under the control of the *tac* promoter. Localization of EaeA to the outer membrane of the vector strains was demonstrated both by Western blotting and by immunofluorescence with an anti-EaeA antibody. A rabbit model for colonization by *V. cholerae* was used to compare the immune responses to the two heterologous antigens, StxB1 and EaeA, expressed by these strains. Rabbits immunized with Peru2 transformed with a plasmid carrying *tac*→*stxB*₁ developed neutralizing serum anti-StxB1 immunoglobulin G antibody responses. One of two rabbits immunized with a strain carrying a chromosomal copy of *eaeA* developed a marked immune response against EaeA. The plasmid construct containing *hspG*→*stxB*₁,*eaeA* was unstable, producing low levels of StxB1 in vitro and not evoking anti-EaeA antibody responses in vivo following oral immunization. Chromosomal insertion of *eaeA* may be preferred for future expression of this antigen in *V. cholerae* vaccine constructs.

The recognition of morbidity caused by enterohemorrhagic *Escherichia coli* (EHEC) strains has increased in recent years, with well-documented outbreaks in the Pacific Northwest of the United States and in Japan (4, 23, 46). These bacteria have been associated with the clinical syndromes of hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (47). In the United States, pathogenic EHEC strains often belong to the serotype O157:H7, but many other serotypes can cause disease (47). Pathogenic strains synthesize Shiga toxin 1 (Stx1) and/or Stx2 (formerly known as Shiga-like toxins I and II, respectively), heterodimeric proteins consisting of five B (binding) subunits and one A (catalytic) subunit, which acts by cleaving a specific adenine residue in the 60S ribosomal subunit of a eukaryotic cell and thus disrupting protein synthesis (50). These cytotoxins appear to be major virulence factors for these organisms.

Although Stx1 from EHEC and Shiga toxin from *Shigella dysenteriae* 1 are poorly immunogenic in patients diagnosed with hemolytic uremic syndrome and *S. dysenteriae* 1 dysentery, respectively (22, 35), serum antibodies against the toxins appear to be protective against toxin-induced disease in animal models (21, 52). Antibodies directed against the nontoxic B

subunit of Stx1 (StxB1) or against synthetic peptides based on its sequence protect against the effects of holotoxin (5, 18, 25, 26, 41). StxB1 has therefore been of particular interest as a component for inclusion in potential EHEC vaccines.

EHEC strains are noninvasive, mucosal pathogens and as such are attractive targets for vaccine strategies that stimulate a mucosal immune response. We have developed live, oral attenuated strains of *Vibrio cholerae* as vaccine vectors that can deliver heterologous antigens to the gut mucosa to evoke antibody responses (6, 7). We have previously used StxB1 as a model heterologous antigen for expression from *V. cholerae* vector strains, and we have demonstrated anti-StxB1 serum immunoglobulin G (IgG) and mucosal IgA antibody responses following oral inoculation of rabbits with *V. cholerae* strains expressing *stxB*₁ from in vivo-expressed promoters on the chromosome (6). Anti-StxB1 antibody production was enhanced when *V. cholerae* strains expressed *stxB*₁ from a constitutive promoter on a high-copy-number plasmid; such antibodies were found to neutralize holotoxin in a cytotoxicity assay (2).

Another potential protective immunogen in EHEC is EaeA, or intimin, a 97-kDa outer membrane protein that mediates attachment to intestinal epithelial cells and subsequent effacement of microvilli (15, 36). *eaeA* was originally recognized as a locus in enteropathogenic *E. coli* (EPEC) which was necessary for intimate attachment of bacteria to epithelial cells in vitro (31). The *eaeA* locus was found to be part of a gene cluster within a 35-kb locus that is present in all bacteria which elicit

* Corresponding author. Mailing address: Infectious Disease Unit, Massachusetts General Hospital, Boston, MA 02114. Phone: (617) 726-3818. Fax: (617) 726-7416. E-mail: ButtertonJ@A1.mgh.harvard.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype ^a	Reference or source
<i>V. cholerae</i> O1 strains		
C6709	El Tor, Inaba, wild-type, Sm ^r	43
Peru2	C6709 $\Delta attRS1$ Sm ^r	43
JRB10	Peru2 $\Delta irgA$ <i>irgA::irgP htpGp</i> → <i>stxB</i> ₁ Sm ^r	6
JRB11	Peru2 <i>lacZ::htpGp</i> → <i>stxB</i> ₁ Sm ^r	6
JRB12	JRB10 <i>lacZ::htpGp</i> → <i>stxB</i> ₁ Sm ^r	6
JRB13	Peru2 <i>lacZ::htpGp</i> → <i>stxB</i> ₁ , <i>eaeA</i> Sm ^r	This study
JRB14	JRB10 <i>lacZ::htpGp</i> → <i>stxB</i> ₁ , <i>eaeA</i> Sm ^r	This study
<i>E. coli</i> strains		
DH5 α	F [−] <i>endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1</i> Δ (<i>argF-lacZYA</i>) <i>U169</i> (ϕ 80d <i>lacZ</i> Δ M15)	Gibco BRL Life Technologies
EHEC EDL932	O157:H7, produces Stx1 and Stx2	ATCC 43894
EPEC 2348/69	Prototypic O127:H6 strain	32
Plasmids		
pSBC54	pKK233-2 with <i>stxB</i> ₁ cloned between the <i>Nco</i> I and <i>Hind</i> III sites downstream of the <i>tac</i> promoter, Ap ^r	1
p6891MCS	pBR327 containing an 8-kbp <i>Sau</i> 3A fragment of <i>V. cholerae</i> DNA encoding <i>lacZ</i> , and with a multiple cloning site inserted at <i>Kpn</i> I within <i>lacZ</i> , Ap ^r	6
pJRB32	1,066-bp <i>Bam</i> HI- <i>Sma</i> I fragment containing <i>htpGp</i> → <i>stxB</i> ₁ , cloned in <i>Bgl</i> II and <i>Stu</i> I sites of p6891MCS, Ap ^r	6
pCVD444	pUC19 with <i>eaeA</i> from EHEC strain EDL932 on a 3.8-kbp <i>Xho</i> II fragment cloned into the <i>Bam</i> HI site, Ap ^r	54
pJRB33	pJRB32 with the <i>Not</i> I site downstream of <i>stxB</i> ₁ replaced by <i>Bgl</i> II	This study
pJRB34	pJRB33 with the 3.8-kbp <i>Xho</i> II fragment from pCVD444 encoding <i>eaeA</i> , cloned into the <i>Bgl</i> II site downstream of <i>stxB</i> ₁ , Ap ^r	This study
pJRB43	4.8-kbp <i>Kpn</i> I fragment of pJRB34 containing <i>htpGp</i> → <i>stxB</i> ₁ , <i>eaeA</i> cloned into the <i>Kpn</i> I site of pUC18, Ap ^r	This study

^a Ap^r, ampicillin resistance; Sm^r, streptomycin resistance.

the attaching-and-effacing phenotype (37). EPEC EaeA is a 94-kDa outer membrane protein which is immunogenic, being recognized by sera from volunteers after EPEC infection (30). A volunteer study with an EPEC *eaeA* mutant demonstrated that EaeA is required for full virulence, although mild diarrhea can still develop in its absence (13, 14). Sequences homologous to EPEC *eaeA* were found on the chromosomes of EHEC strains (29), allowing the cloning and sequencing of EHEC *eaeA* (3, 54). The amino acid sequences of EPEC EaeA and EHEC EaeA are 83% identical and have homology with the invasins of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (54). EHEC EaeA, a 97-kDa outer membrane protein, is required for attachment of EHEC to cells in tissue culture and for formation of the attaching-and-effacing lesion in animal models (15, 38, 51). Anti-EaeA-specific antisera, raised against a histidine-EaeA fusion protein, recognized native EaeA in both EHEC and EPEC and prevented the attachment of EHEC to HEP-2 cells (39).

The role of EaeA as a virulence factor, its immunogenicity, and the ability of anti-EaeA antisera to block adherence of EHEC to epithelial cells suggest the use of this antigen as a potential vaccine component. We had previously placed *stxB*₁ under the transcriptional control of the *V. cholerae* heat shock promoter *htpGp* (6). In the present study, a chromosomal EHEC fragment containing *eaeA* was added to the construct, downstream of *stxB*₁. The *htpGp*→*stxB*₁,*eaeA* construct was inserted into the *V. cholerae* chromosome by in vivo marker exchange, and the resulting vector strains were analyzed. Strains were constructed to allow a comparison not only of the expression of *stxB*₁ under control of either constitutive or in vivo-expressed promoters but also of the difference in immunological effect when *stxB*₁ and *eaeA* were expressed either from a chromosomal locus or from a plasmid.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. The standard plasmid cloning vector pUC18 was from laboratory stock.

Media. All strains were maintained at −70°C in Luria broth (LB) medium (44) containing 15% glycerol. LB medium or AKI medium (28) was used for bacterial growth. Ampicillin (100 µg/ml), streptomycin (100 µg/ml), or X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (International Biotechnologies, Inc., New Haven, Conn.) (80 µg/ml) was added as appropriate.

Genetic methods. Isolation of plasmid and bacterial chromosomal DNAs, restriction enzyme digests, agarose gel electrophoresis, and Southern hybridization of DNA separated by electrophoresis were performed by standard molecular biologic techniques (44). GeneScreen Plus hybridization transfer membranes (DuPont Biotechnology Systems, NEN Research Products, Boston, Mass.) were used according to the manufacturer's protocols for Southern hybridization. DNA sequencing was performed by using the Sequenase DNA Sequencing Kit (United States Biochemical Corporation, Cleveland, Ohio). Bacterial cell-free coupled transcription-translation was performed with the Prokaryotic DNA-Directed Translation Kit (Amersham Corporation, Arlington Heights, Ill.). A 2.5-µg amount of plasmid DNA was used per reaction. Radio-labelled protein products were separated on a 0.1% sodium dodecyl sulfate

TABLE 2. Production of StxB1 as measured by ELISA in periplasmic extracts from *V. cholerae* strains

Strain	StxB1 (ng/ml/OD ₆₀₀ unit) ^a
JRB10.....	27 (15–51)
JRB11.....	7 (6–9)
JRB12.....	52 (50–55)
JRB13.....	13 (10–17)
JRB14.....	78 (38–117)
Peru2.....	0
Peru2(pJRB43).....	6 (1–10)
Peru2(pSBC54).....	458 (103–812)

^a Mean (range) of two independent measurements. OD₆₀₀, optical density at 600 nm.

FIG. 1. In vitro transcription-translation. Lane 1, pJRB32 DNA; lane 2, pJRB34 DNA; lane 3, control without DNA. The numbers on the left indicate the molecular masses (in kilodaltons) of the protein standards. The arrow points to a unique protein product synthesized by pJRB34 with a molecular mass of approximately 96 kDa, consistent with EaeA.

(SDS)-12.5% polyacrylamide gel and autoradiographed by standard techniques (44).

Plasmids were transformed into *E. coli* strains by standard techniques or were electroporated into *V. cholerae* by using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.), following the manufacturer's protocol, and modified for electroporation into *V. cholerae* as previously described (19). Electroporation conditions were 2,500 V at a 25- μ F capacitance, producing time constants of 4.7 to 4.9 ms.

DNA restriction endonucleases, T4 DNA ligase, calf intestinal alkaline phosphatase, and the Klenow fragment of DNA polymerase I were used according to the manufacturer's specifications. Restriction enzyme-digested chromosomal and plasmid DNA fragments were separated on 1% agarose gels; required fragments were cut from the gel under UV illumination and purified by the freeze-squeeze technique (48). DNA fragments used as probes were radiolabelled with [α - 32 P]dCTP by using a random priming labelling kit (Prime Time "C" Oligonucleotide Labelling Biosystem; International Biotechnologies, Inc.).

Construction of plasmids. Plasmid pJRB32 contains *stxB1* under the control of the *V. cholerae* heat shock promoter *htpGp*, within *V. cholerae lacZ* on the plasmid vector p6891MCS (6). Plasmid pCVD444 contains *eaeA* from EHEC strain EDL932 on a 3.8-kbp *Xho*II fragment cloned into the *Bam*HI site of pUC19 (54). The unique *Not*I site in pJRB32, which lies immediately down-

stream of *stxB1*, was replaced with a *Bgl*II site to create pJRB33. The 3.8-kbp *Xho*II fragment from pCVD444 was inserted in the *Bgl*II site of pJRB33 to create pJRB34. Transcription of *eaeA* in pJRB34 is in the same orientation as that of *stxB1*, as verified by restriction enzyme digestion and double-stranded DNA sequencing.

Introduction of pJRB34 into *V. cholerae* always resulted in immediate marker exchange between the chromosomal *lacZ* locus and the *lacZ* sequences flanking *htpGp*→*stxB1*,*eaeA* on the plasmid (see "Construction of JRB13 and JRB14" below); the synthesis of large amounts of EaeA from a high-copy-number plasmid in *V. cholerae* may create selective pressure for such a recombination event. In order to express *htpGp*→*stxB1*,*eaeA* from a high-copy-number plasmid in *V. cholerae*, the 4.8-kbp *Kpn*I fragment from pJRB34 was recloned into the unique *Kpn*I site of pUC18 to yield plasmid pJRB43. Transcription of *htpGp*→*stxB1*,*eaeA* is in the opposite orientation to the *lacZ* promoter, as confirmed by restriction enzyme digestion.

Construction of JRB13 and JRB14. Plasmid pJRB34 contains *htpGp*→*stxB1*,*eaeA* cloned within a deletion of *V. cholerae lacZ*. *V. cholerae* strains were transformed with this plasmid by electroporation, grown overnight in the absence of ampicillin selection, and then plated on medium containing X-Gal. Strains undergoing marker exchange between pJRB34 and the chromosomal copy of *lacZ* were white on X-Gal medium and ampicillin susceptible. By using this procedure, *htpGp*→*stxB1*,*eaeA* was inserted into *lacZ* on the chromosomes of Peru2 (49) and JRB10 (6) to create JRB13 and JRB14, respectively. The expected insertions in *lacZ* within JRB13 and JRB14 were confirmed by Southern hybridization (data not shown).

Preparation of outer membrane proteins, whole-cell proteins, and periplasmic extracts. Enriched outer membrane proteins, whole-cell proteins, and periplasmic extracts were prepared from exponentially growing cells as previously described (20, 27). *V. cholerae* strains were grown in AKI medium, and *E. coli* strains were grown in LB medium.

Immunodetection of StxB production. The amount of StxB1 present in periplasmic extracts was quantitated with an enzyme-linked immunosorbent assay (ELISA) developed for the detection of Shiga toxin and modified for detection of purified StxB1 (9, 17).

Immunodetection and localization of synthesized EaeA. Whole-cell proteins and enriched outer membrane proteins were separated by electrophoresis on SDS-7.5% polyacrylamide gels and then transferred to NitroBind Transfer Membranes (Micron Separations Inc., Westboro, Mass.) with a semidry blotting apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). Immunoreactive proteins were visualized after sequential incubation with polyclonal rabbit anti-EaeA antiserum (39) and goat anti-rabbit IgG-conjugated alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.), followed by staining for phosphatase activity as described previously (27).

Immunofluorescence assays were performed as follows. *V. cholerae* JRB11 and JRB13 were grown to late log phase and diluted to 10^7 organisms per ml in phosphate-buffered saline (PBS). Three hundred microliters of each dilution was spun onto coverslips in 24-well plates. Cells were fixed with 500 μ l of 4% paraformaldehyde per well and then washed three times with PBS and one time with PBS-2% goat serum. Cells were then incubated overnight with rabbit polyclonal antiserum against EaeA, diluted 1:1,000 in PBS-2% goat serum. Following three washes with PBS-2% goat serum, cells were incubated with goat anti-rabbit IgG fluorescein conjugate (Sigma) diluted 1:500 in PBS-goat serum. Coverslips were stained for 5 min with 0.1 μ g of 4',6'-diamidino-2-phenylindole (DAPI) (Sigma) per ml diluted in PBS and were mounted for microscopy for detection of fluorescein and DAPI.

Orogastric colonization of rabbits. Orogastric colonization of rabbits was performed as previously described (11), with the following modifications. *V. cholerae* strains were grown overnight in LB medium, pelleted, washed in PBS, and resuspended in fresh LB medium to a concentration of 10^9 organisms per ml. Male New Zealand White rabbits (approximately 2.5 kg) were fasted overnight and sedated. Gastric acid was neutralized with 50 mg of cimetidine per kg (intravenously) at time zero. Ten milliliters of 5.0% NaHCO₃ was given by gastric tube at 15 and 30 min; the second dose was followed by the *V. cholerae* inoculum in 10 ml of LB medium. At 60 min, 1 ml of tincture of opium was given intraperitoneally. Oral inoculations were repeated on day 14. Serum was drawn on days 0, 14, and 28; bile was obtained on day 28 to estimate mucosal secretory IgA. Two rabbits each were inoculated with Peru2 and JRB14; three rabbits each were inoculated with Peru2(pSBC54) and Peru2(pJRB43).

Rabbit serum 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 at 56°C for 1 h. Fifty-microliter amounts of serial twofold dilutions (1:25 to 1:25,600) of the test sera in PBS were placed in the wells of sterile 96-well tissue culture plates. Fifty microliters of a 10^8 -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 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 incubated for approximately 2.75 h at 37°C. The optical density at 600 nm was then measured; the titer was calculated as the dilution of serum causing a 50% reduction in optical density compared with wells containing preimmune serum (day 0).

FIG. 2. Reactivity of polyclonal anti-EaeA antiserum (1:1,000) with proteins from *V. cholerae* and *E. coli* strains. (A) Whole-cell proteins. Lane 1, purified recombinant histidine-EaeA fusion protein; lane 2, Peru2(pJRB43). The numbers on the right indicate the molecular masses (in kilodaltons) of the protein standards. (B) Outer membrane proteins. Lane 1, JRB13; lane 2, JRB11; lane 3, EHEC EDL932; lane 4, EPEC 2348/69; lane 5, DH5 α . The numbers on the right indicate the molecular masses (in kilodaltons) of the protein standards. The arrows indicate the 94- and 75-kDa immunoreactive proteins in the outer membrane fraction of JRB13, consistent with nearly full-length EaeA (97 kDa [lane 3]) as well as a truncated breakdown product. The 94- and 75-kDa immunoreactive proteins were also detected in the outer membrane fraction of JRB14 (data not shown).

FIG. 3. Immunofluorescence assays. *V. cholerae* strains were incubated with rabbit polyclonal antiserum against EaeA (1:1,000) followed by goat anti-rabbit IgG fluorescein conjugate (1:500) and stained with DAPI. See text for details. (A) JRB11 (DAPI); (B) JRB11 (fluorescein); (C) JRB13 (DAPI); (D) JRB13 (fluorescein).

Rabbit serum and mucosal antibodies to StxB1. Microtiter plates (Nunc Maxisorp) were coated with mouse monoclonal anti-StxB1 antibody, incubated with 1 μ g of purified StxB1 (1) in carbonate buffer (pH 9.6) per ml, and then blocked with PBS–1% bovine serum albumin. Duplicate 1:50 dilutions of serum samples and 1:40 dilutions of bile samples in PBS–0.05% Tween were added to the microtiter wells. Serum anti-StxB1 IgG was assayed by using a 1:4,000 goat anti-rabbit IgG–horseradish peroxidase conjugate (Southern Biotechnology Associates, Inc., Birmingham, Ala.) in PBS–0.05% Tween. Reactions were developed with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma) with 0.1% H_2O_2 (Sigma), and the optical density at 405 nm was measured kinetically with a Molecular Devices Vmax kinetic microplate reader. Plates were read for 5 min at 11-s intervals to a maximum optical density of 1.5; results are expressed as milli-optical density units per minute. Bile anti-StxB1 IgA was determined by using goat antiserum against rabbit IgA conjugated to horseradish peroxidase (Nordic Immunological Laboratories, San Clemente, Calif.) and then ABTS- H_2O_2 as described above.

Rabbit serum and mucosal antibodies to EaeA. Microtiter plates were coated with 0.5 μ g of recombinant histidine-EaeA fusion protein (containing 900 of the 935 amino acids encoded by *eaeA* from EHEC O157:H7 strain 86-24 [39]) per ml and then blocked with PBS–1% bovine serum albumin. Duplicate serial twofold dilutions of serum or bile samples in PBS–0.05% Tween were added to the microtiter wells. Serum anti-EaeA IgG was assayed by using goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma). Bile anti-EaeA IgA was determined by using goat antiserum against rabbit IgA (alpha chain specific) (Sigma) and then rabbit anti-goat IgG conjugated to alkaline phosphatase (Sigma). Reactions were developed with 2 mg of *p*-nitrophenyl phosphate per ml diluted in 1 M Tris buffer (pH 8.0), and the results were expressed as optical density at 405 nm.

The recombinant histidine-EaeA fusion protein (1.5 μ g/well) was separated by SDS–7.5% polyacrylamide gel electrophoresis and then transferred to membranes as described above. Immunoreactive proteins were visualized after sequential incubation with pre- or postimmunization rabbit sera, followed by goat anti-rabbit IgG-conjugated alkaline phosphatase and staining for phosphatase activity as described above. Rabbit polyclonal anti-EaeA serum was used as a positive control.

Neutralization of Vero cell cytotoxicity. The ability of test sera to neutralize the cytotoxicity of Stx1 was determined by measuring the incorporation of [3H]leucine into protein by Vero cells (16). Various concentrations of serum were incubated with Stx1 (1 ng/ml) for 1 h at room temperature before the toxin-serum mixture was added to the Vero cells. The results were expressed as percent leucine incorporation by Vero cells compared with that by non-toxin-treated control cells.

RESULTS

Synthesis of StxB1 by *V. cholerae* vector strains in vitro. The synthesis of StxB1 in vector strains was quantified by using a sandwich ELISA to determine StxB1 concentrations in periplasmic extracts following late-log-phase growth of *V. cholerae* strains in AKI medium. As shown in Table 2, in vitro synthesis of StxB1 by JRB13 and JRB14 was comparable to that by JRB11 and JRB12, respectively, indicating that the insertion of *eaeA* downstream of *htpGp*→*stxB*₁ did not alter expression from the heat shock promoter. The synthesis of StxB1 from Peru2 containing the plasmid pJRB43 was substantially lower than expected, and this correlated with difficulty in detecting the plasmid in DNA preparations; toxicity from high-level expression of *eaeA* may have selected against plasmid stability. Peru2(pSBC54), in which *stxB*₁ was expressed from the *tac* promoter (without *eaeA*), produced the greatest amount of StxB1 in vitro.

Synthesis and localization of EaeA in *V. cholerae* strains. The protein products of the cloned insert in pJRB34 were analyzed by using a bacterial cell-free coupled transcription-

FIG. 4. Vibriocidal antibody titers following oral inoculation of rabbits with various strains of *V. cholerae*. See text for details. GMT, geometric mean titer. Error bars depict the standard errors of the mean.

translation system. As shown in Fig. 1, the major unique protein product synthesized from pJRB34 had a molecular mass of approximately 96 kDa, in agreement with the predicted molecular mass of EaeA.

Whole-cell proteins were prepared from an overnight growth of Peru2(pJRB43), and immunoreactive proteins were visualized by Western immunoblotting with polyclonal anti-EaeA antiserum. As shown in Fig. 2A, a protein of approximately 95 kDa was recognized in the whole-cell preparation, suggesting production of nearly full-length EaeA by the plasmid construct. Synthesis and localization of EaeA by strains JRB13 and JRB14 were evaluated by Western immunoblotting for periplasmic extracts and enriched outer membrane proteins of these strains. Immunoreactive proteins of approximately 94 and 75 kDa were recognized in outer membrane fractions of JRB13 (Fig. 2B) and JRB14 (data not shown) but not in periplasmic extracts from these strains (data not shown). These two proteins most likely represent nearly full-length EaeA, as well as a truncated breakdown product, similar to the breakdown products seen with purified recombinant histidine-EaeA fusion protein (Fig. 2A) (39). The localization of EaeA on the surface of JRB13 was confirmed by an immunofluorescence assay with whole bacteria (Fig. 3).

Serum vibriocidal responses following oral immunization of rabbits. Successful oral immunization of rabbits with various *V. cholerae* strains was demonstrated by high serum vibriocidal antibody responses on days 14 and 28 postinoculation (Fig. 4).

Serum IgG and bile IgA antibody responses to StxB1 produced by *V. cholerae* vector strains. The immune responses to StxB1 synthesized by the vector strains were also evaluated following oral immunization of rabbits. Immunization with JRB14 and Peru2(pJRB43) failed to provoke detectable serum anti-StxB1-specific IgG antibodies, but the rabbits immunized with

Peru2(pSBC54) had measurable anti-StxB1 IgG antibody responses on day 28, after two doses of vaccine (Fig. 5). None of the three vector strains evoked biliary IgA responses specific for StxB1 (data not shown). The absence of measurable serum anti-StxB1 IgG in response to immunization with JRB14 and Peru2(pJRB43) is consistent with the low levels of expression of StxB1 from these strains observed *in vitro* (Table 2). Serum anti-StxB1 antibodies following immunization with Peru2(pSBC54) were protective, neutralizing the cytotoxic effects of Stx1 for Vero cells (Fig. 6).

Serum IgG and bile IgA antibody responses to EaeA expressed by *V. cholerae* vector strains. Sera from immunized animals were initially screened for anti-EaeA IgG antibody responses by ELISA. One of two rabbits immunized with JRB14 developed a marked immune response against EaeA as measured by ELISA; no animals developed anti-EaeA IgA antibody responses (data not shown). The specificity of the serum IgG antibody response was demonstrated by Western blotting, which showed that day 28 serum at a concentration of 1:5,000 (but not preimmune serum) recognized the recombinant histidine-EaeA fusion protein (Fig. 7). The animals immunized with Peru2(pJRB43) did not develop detectable antibody responses to EaeA, consistent with the absence of antibody responses to StxB1 and the observed instability of this plasmid construct in *V. cholerae*.

DISCUSSION

V. cholerae has many characteristics that make it a particularly effective delivery vehicle for heterologous antigens (6–8). Human infection with *V. cholerae* leads to prolonged colonization (7 to 14 days), inducing excellent mucosal and systemic humoral immunity (33); organisms selectively attach to intes-

FIG. 5. Anti-StxB1 IgG ELISA results for serum at days 0, 14, and 28 after oral inoculation of the indicated strains. Duplicate 1:50 dilutions of serum were analyzed by ELISA, and the optical density at 405 nm was measured kinetically for 5 min at 11-s intervals. mOD, milli-optical density units. Results are means $\pm$ standard errors of the means for the rabbits in each group.

tinal M cells, presenting antigens directly to lymphoid tissue and producing strong mucosal immune responses (12, 42, 45). Live oral attenuated cholera vaccines that can be used to synthesize foreign antigens have already been developed (10, 34, 40, 49, 53). *V. cholerae* has a large genomic capacity, allowing the incorporation of substantial amounts of heterologous DNA, and methods for inserting this DNA into the chromosome of *V. cholerae* by in vivo marker exchange are well established. A number of in vivo active promoters have been identified, enhancing synthesis of foreign antigens after inoculation with *V. cholerae*. Finally, animal models are available for assessing immune responses to orally administered *V. cholerae* vectors.

This study demonstrates the use of *V. cholerae* as a vector to deliver two distinct antigens from EHEC to the gut mucosa to induce systemic immune responses. We describe the insertion into the *V. cholerae* chromosome and subsequent expression of EHEC StxB1 and EaeA, two antigens which are known virulence factors, which are immunogenic, and antibodies to which are protective against disease caused by this organism. The antigens are inserted within *lacZ* on the *V. cholerae* chromosome by a recently described method of in vivo marker exchange (6). Each antigen localizes to a different cell compartment; StxB1 remains in the periplasm, while EaeA is inserted in the outer membrane. Both proteins are recognized by specific antisera and so appear to have retained their native immunological epitopes.

A previous study demonstrated the ability of a classical *V. cholerae* vaccine strain, CVD 103-HgR (34), containing a plasmid vector carrying *stxB*₁ under the control of a *tac* promoter to evoke serum anti-StxB1 IgG antibodies in a rabbit model; sera from these animals also neutralized Stx1 in a cytotoxicity

assay (2). In this study, rabbits inoculated with a *V. cholerae* El Tor strain expressing *stxB*₁ from a *tac* promoter on a plasmid vector [Peru2(pSBC54)] also produced a neutralizing serum anti-StxB1 IgG response. A plasmid construct containing *stxB*₁, *eaeA* under the control of the *V. cholerae* heat shock promoter *htpGp* [Peru2(pJRB43)] was also evaluated. EaeA was expressed from this strain when the plasmid was maintained in the *V. cholerae* vector with antibiotic pressure in vitro, and it was localized to the outer membrane. However, markedly less StxB1 was produced in vitro in periplasmic extracts from Peru2(pJRB43) than from Peru2(pSBC54), and no anti-StxB1 or anti-EaeA IgG antibodies were detectable following inoculation of rabbits with Peru2(pJRB43). These results are consistent with the instability of pJRB43 in the *V. cholerae* host and with the more rapid loss of pJRB43 than of pSBC54 after inoculation into the antibiotic-free environment of the rabbit gut.

Given that our inability to evoke anti-EaeA immune responses with *V. cholerae* carrying *eaeA* on a plasmid vector might be due to instability of the plasmid, we proceeded to place *eaeA* on the *V. cholerae* chromosome in tandem with *stxB*₁ to allow stable synthesis of these antigens. Both JRB13 and JRB14 produced substantially less StxB1 in vitro than did the strains synthesizing StxB1 from plasmid vectors, although JRB14 produced more StxB1 in periplasmic extracts in vitro than did JRB13, suggesting that production correlates with copy number. Both JRB13 and JRB14 synthesized EaeA and localized this antigen to their outer membranes, where it was recognized by specific anti-EaeA antisera. Serum anti-EaeA IgG antibodies were detected in one of two rabbits following oral inoculation with JRB14, suggesting that expression of EaeA by this vector is sufficient for the development of an

FIG. 6. Neutralization of Stx1 cytotoxicity for Vero cells after preincubation with dilutions of sera from the two control animals inoculated with Peru2 and the three animals given Peru2(pSBC54). Results are means $\pm$ standard errors of the means for each group. Vero cell survival is calculated as a percentage of the leucine incorporation for cells grown in the absence of toxin (high incorporation values denote low levels of toxin activity).

immune response to this antigen. However, we did not detect an anti-StxB1 antibody response in these rabbits. In a previous study, we detected low levels of serum IgG and biliary IgA anti-StxB1 antibody responses on day 21 following vaccination of rabbits with vector strains JRB10, JRB11, and JRB12 (6); we were surprised, therefore, that vaccination with JRB14 did not yield an anti-StxB1 response. It is uncertain whether this difference relates to the fact that JRB14 synthesizes EaeA in addition to StxB1 (and synthesis of this heterologous outer membrane protein may slow growth), to the fact we assayed for anti-StxB1 antibody responses on days 14 and 28 in this study compared with day 21 in the previous study, or to other factors not identified.

FIG. 7. Reactivity of rabbit serum (1:5,000) with purified recombinant histidine-EaeA fusion protein. Lane 1, preimmune rabbit serum; lane 2, serum from the same rabbit at day 28 after oral inoculation with JRB14; lane 3, polyclonal anti-EaeA antiserum. The numbers on the right indicate the molecular masses (in kilodaltons) of the protein standards.

One tactic for increasing antibody responses to heterologous antigens is to give repeated oral doses of the vector strains. Rather than inducing tolerance, it has been observed that immune responses to heterologous antigens carried by *V. cholerae* have been significantly boosted by second oral immunizations (2, 24). In this study, rabbits received two oral inoculations of the *V. cholerae* vector strains 2 weeks apart. No clear boosting of vibriocidal immune responses was observed between days 14 and 28 following the initial oral dose, but serum anti-StxB1 IgG antibodies, which were not present at day 14 following oral immunization, were detectable at day 28.

Our results suggest that *V. cholerae* can function as a delivery vehicle for multiple heterologous antigens simultaneously and can present immunogenic molecules in a variety of cell compartments. Antigens presented along with immunogenic molecules, such as outer membrane proteins that are presented to the mucosal immune system along with native lipopolysaccharide, may be the most effective in evoking immune responses. Antigens that are secreted or those that have high native immunogenicity, such as the B subunit of cholera toxin, also work well in this delivery system (6). Periplasmic proteins such as StxB1 may need to be delivered at high concentrations—under the control of constitutive promoters and on high-copy-number plasmids—in order to produce substantial antibody responses. It would be preferable for vaccine development to have genes encoding heterologous antigens stably inserted into the chromosome of a vector, rather than carried on a plasmid, to avoid the potential loss of the genes during bacterial replication. Methods to enhance the expression and delivery of

chromosomally encoded foreign antigens in *V. cholerae* vectors are currently being pursued.

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