INFECTION AND IMMUNITY, June 1996, p. 2144–2150 Vol. 64, No. 6
0019-9567/96/$04.00+0

Synthesis of Hybrid Molecules between Heat-Labile Enterotoxin and Cholera Toxin B Subunits: Potential for Use in a Broad-Spectrum Vaccine

M. LEBENS,* V. SHAHABI, M. BÄCKSTRÖM, T. HOUZE, M. LINDBLAD, AND J. HOLMGREN

Department of Medical Microbiology and Immunology, University of Göteborg, S-413 46 Göteborg, Sweden

Received 22 November 1995/Returned for modification 15 January 1996/Accepted 12 March 1996

Three variants of the cholera toxin B subunit (CTB) were generated by site-specific mutagenesis in which regions of the mature protein were altered to the composition found at the corresponding positions of the closely related B subunit of the heat-labile enterotoxin of enterotoxigenic *Escherichia coli* (LTB). The mutant proteins were expressed in *Vibrio cholerae* and purified from the growth medium. In the first of the mutant proteins, the first 25 amino acids corresponded to the sequence found in LTB, and in the second, changes were made at positions 94 and 95 of the mature protein. The third mutant protein combined the changes made in the first two. Analysis of the immunological properties of these novel proteins by using monoclonal antibodies and absorbed polyclonal antiserum demonstrated that they had acquired LTB-specific epitopes. Immunizations with the mutant proteins resulted in antisera containing LTB-specific as well as CTB-specific and cross-reactive antibodies. The sera were also found to be more strongly cross-reactive in the in vitro neutralization of both cholera toxin and heat-labile enterotoxin than were antisera raised against either CTB or LTB. The results suggest that such hybrid CTB-LTB proteins may be useful in a broad-spectrum vaccine against enterotoxin-induced diarrhea.

Cholera remains an important cause of illness in many developing countries and has been estimated to result in more than 120,000 deaths each year (10). Infection with enterotoxigenic *Escherichia coli* (ETEC) is the most frequent cause of diarrhea in the developing world and among travelers; among children alone, it is responsible for more than 650 million diarrheal episodes and 800,000 deaths annually in developing countries (2). There is a great need for effective vaccines against both cholera and ETEC infections.

In both infections, the primary cause of diarrhea is the action of an enterotoxin released by the infecting organisms in the intestine: in the case of cholera, cholera toxin (CT), and in the case of ETEC, heat-labile enterotoxin (LT). The two toxins are closely related both structurally and functionally, each consisting of a toxic A subunit (CTA or LTA, respectively) surrounded by five identical B subunits (CTB or LTB, respectively) (24). The B-subunit pentamers are responsible for the binding of the toxin to ganglioside M1 (GM1) receptors present on the surface of intestinal epithelial cells (7); LT can also bind to structurally related galactoprotein receptors (8).

LTB and CTB show a high degree of homology, with 85% conservation of amino acids in the mature protein (5) (Table 1), and there is evidence from crystallographic studies that LTB and CTB pentamers are also structurally similar (15, 22, 23). There is also a high degree of immunological cross-reactivity between the two molecules (29) despite the fact that the majority of antibodies are directed against structural features of assembled pentamers, a further indication of the structural similarity between the two molecules.

CT has been found to be an effective oral immunogen that gives rise to intestinal immunoglobulin A (IgA) responses directed mainly against the B subunit. Furthermore, oral administration of CTB alone has also been found to effectively stimulate similar responses, and especially in humans, CTB has been found to be a strong immunogen in the absence of either the adjuvant or toxic effects of the holotoxin. These responses are associated with high-level although relatively short-term (ca. 6 months) protection against challenge or natural infection with *Vibrio cholerae* and a much longer-lasting immunological memory (17, 26, 30, 31).

Antitoxin antibodies appear to act synergistically together with intestinal IgA antibodies directed against bacterial cell-associated antigens such as the lipopolysaccharide (LPS) of *V. cholerae* O1 (27) or of the novel serotype O139 (7a). On the basis of these findings, an oral vaccine against cholera, consisting of CTB together with killed whole cells of *V. cholerae* O1, has been developed (9) and has given rise to protection lasting several years (4); and this vaccine is presently being modified to also include cells of the O139 serotype.

Large-scale field trials of the B subunit-O1 whole cell vaccine in Bangladesh demonstrated, in addition to long-term protection against cholera, significant short-term cross-protection against ETEC infection due to the CTB component (3). Such protection against ETEC afforded by the cholera vaccine was subsequently confirmed in a study of Finnish tourists traveling to Morocco (16). On the basis of this finding, a broader-spectrum vaccine against ETEC in which CTB is used in conjunction with killed *E. coli* expressing the major colonization factor antigens involved in adhesion to the intestinal epithelium has been developed (25). The *E. coli* strains were included in order to provide immunity against ETEC strains releasing a heat-stable enterotoxin (STa) either alone or together with LT.

In areas where both cholera and ETEC are endemic, it would be desirable to have a single vaccine that could effectively protect against both infections. This could in part be achieved by increasing the protection against ETEC afforded by the CTB component of the licensed cholera vaccine. Although LTB and CTB show significant immunological cross-reactivity, serum from CTB-immunized individuals does not

* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, University of Göteborg, Guldhedsgatan, 10A, S-413 46 Göteborg, Sweden. Phone: 46-31-604720. Fax: 46-31-820160.

TABLE 1. Comparison between the sequences of the mature human LTB and mature CTB used in this study

Position	Amino acid				
	rCTB[a]	LTB[b]	LCTB*A*	LCTB*B*	LCTB*H*
1	Ala	Ala	Ala	Ala	Ala
4	Asn	Ser	Ser		Ser
7	Asp	Glu	Glu		Glu
10	Ala	Ser	Ser		Ser
18	His	Tyr	Tyr		Tyr
20	Ile	Ile	Ile		Ile
25	Phe	Leu	Leu		Leu
31	Leu	Met			
38	Ala	Val			
44	Asn	Ser			
75	Ala	Thr			
80	Ala	Thr			
82	Val	Ile			
83	Glu	Asp			
94	His	Asn		Asn	Asn
94	Ala	Ser		Ser	Ser
102	Ala	Glu			

[a] Sequence of mature CTB based on the sequence of Sanchez and Holmgren (19) in which the mature protein has been modified such that there are no additional residues at the amino terminus and the first amino acid is alanine rather than threonine (13).

[b] Sequence of human LTB based on the sequence of Leong et al. (14).

neutralize LT as effectively as it neutralizes CT (1). Conversely, it is known that antisera against LT or LTB react in higher titer with LT than with CT (29). It is therefore likely that some neutralizing epitopes in LTB are absent in CTB. This idea is further supported by the identification of LTB-specific neutralizing antibodies (32).

The inclusion of CTB in both the cholera and ETEC vaccines has led to the development of an expression system for the overproduction of a recombinant protein that can be obtained in large quantities and in the total absence of the toxic CTA subunit (13, 19). This advance has also opened the way to relatively simple procedures for the genetic modification of CTB, primarily for the generation of protein fusions carrying foreign peptide antigens, but also for modification by site-directed mutagenesis to produce hybrid proteins in which LTB-specific epitopes can be introduced into a protein that remains essentially CTB. The present study aims to produce such hybrid molecules carrying LTB-specific epitopes in addition to cross-reactive and CTB-specific ones in order to increase immunological cross-reactivity. The hybrid molecules used have changes both in a surface-exposed region of the pentamer (residues 1 to 25 of the mature protein) and in a region thought to be involved in the differences in the receptor binding specificities observed between CTB and LTB (residues 94 and 95). A final hybrid protein combines all of these changes in a single molecule. It is hoped that these investigations will result in a B-subunit hybrid that can be used in a broad-spectrum vaccine to combat enterotoxigenic infections.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study for the production of recombinant CTB (rCTB) and LTB (rLTB) and mutant derivatives were the O1 El Tor *V. cholerae* strain JBK70 (11) and the O1 classical *V. cholerae* strain JS1569 (13). Strains carrying the appropriate recombinant plasmids were stored frozen in medium with 20% glycerol at −70°C. Cultures were revived by streaking each strain onto Luria-Bertani agar supplemented with ampicillin (100 μg/ml).

Plasmids generated in this study are shown in Fig. 1.

DNA manipulation. Manipulation of DNA, including plasmid preparation, use of restriction enzymes, DNA ligation, and agarose gel electrophoresis, was done according to standard procedures essentially as described by Sambrook et al. (18). Restriction enzymes (Boehringer Mannheim, Mannheim, Germany) were used in the buffers supplied by the manufacturer. Oligonucleotides for insertion into plasmids by ligation or for amplification by PCR were obtained from Innovagen AB, Lund, Sweden.

PCR was used to introduce mutations into a plasmid-borne *ctxB* gene by the method described by Schödel et al. (21). DNA sequencing was done with a Sequenase version 2.0 kit obtained from Amersham International (Amersham, England). All DNA-modifying enzymes, including *Taq* polymerase, were obtained from Boehringer Mannheim.

Preparation of CTB and LTB and their mutant derivatives. Crude rCTB and its derivatives were prepared from *V. cholerae* JS1569 carrying the relevant expression plasmid. LTB was prepared from *V. cholerae* JBK70 carrying the expression plasmid pMMB68 (20).

Cultures were grown on modified syncase (13), and the CTB or LTB accumulated in the medium was recovered by hexametaphosphate precipitation as described previously (13). The concentration of the B subunit obtained was determined by GM1 enzyme-linked immunosorbent assay (ELISA) (28), using the cross-reactive monoclonal antibody LT39.

A method for further purification LTB and CTB derivatives by high-pressure liquid chromatography (HPLC) was developed. Hexametaphosphate precipitates were resuspended in 20 mM Tris-HCl (pH 9). The resulting suspension was then centrifuged at 10,000 rpm for 30 min, and the supernatant containing the B subunit was filtered through a 0.2-μm-pore-size sterile filter. HPLC was performed on a Waters 600 Multisolvent Delivery System (Millipore Corp., Bedford, Mass.), using a DEAE Mem-Sep HP1000 column (Millipore). The B subunits were first bound to the column at pH 9 (20 mM Tris-HCl) and eluted from the column with 0.16 M (for LCTB*A*) or 0.18 M (for LCTB*B* and LCTB*H*) sodium acetate at pH 8.

In vitro antigenicity and protein analysis. Wells of polystyrene ELISA plates were coated with 100 μl of GM1 (0.3 nmol/ml) for 6 h at room temperature and kept at 4°C until use. Coated plates were first washed with two changes of phosphate-buffered saline, pH 7.2 (PBS), and blocked with PBS–0.1% (wt/vol) bovine serum albumin (BSA) for 30 min at 37°C. Solutions of 250 ng of each B subunit preparation per ml were obtained by diluting concentrated stock solutions in PBS–0.1% BSA. One hundred-microliter aliquots of these diluted samples were used to coat the blocked plates by incubation for 1 h at room temperature.

Different antibody solutions were then added to the first row of plates and titrated out with fivefold serial dilutions. The antibodies bound to the B subunits were then detected with a secondary anti-mouse or anti-rabbit immunoglobulin conjugated with horseradish peroxidase (Jackson Immunoresearch, West Grove, Pa.). After incubation for 1 h at room temperature, the binding of the antibodies was detected by using the chromogenic substrate *o*-phenylenediamine and H_2O_2 in citrate buffer (pH 4.5) as described previously (28). The plates were washed three times with PBS–0.05% Tween between each step. Titers were determined as the reciprocal dilution giving an A_{450} of 0.4 above background after a reaction time of 10 min.

Sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis (PAGE) and electrotransfer of resolved proteins to nitrocellulose were done with a Mini Protean II apparatus obtained from Bio-Rad (Richmond, Calif.) under conditions recommended by the manufacturer.

Mouse immunizations. Groups of five C57/BL mice were immunized three times intraperitoneally (i.p.) with either CTB, LTB, or one of the mutant CTB derivatives. The first two doses contained 10 μg of B subunit administered in 200 μl containing Freund's incomplete adjuvant and were given 10 days apart. The third dose of 20 μg of B subunit was given without any adjuvant 14 days after the second dose. The mice were sacrificed 7 days after the third dose, and blood was collected for analysis. Sera were prepared and stored at −20°C until used.

Additional groups of mice were immunized three times intranasally with either CTB, LTB, or one of the hybrid proteins. Each dose was 20 μg administered without adjuvant in 25 μl of physiological saline solution. The first two doses were given 12 days apart, and the final dose was given 10 days later. Seven days after the final dose, the animals were sacrificed as in the i.p.-immunized groups.

Serum titer determination. Sera obtained from immunizations were tested by GM1 ELISA (28). Immediately before use, GM1-coated plates were first washed twice with PBS and blocked with PBS–0.1% BSA at 37°C for 30 min. The plates were then divided into two groups; 100 μl of CTB (0.5 μg/ml in PBS–0.1% BSA) was added to the wells of the first group of plates, and 100 μl of LTB (0.5 μg/ml in PBS–0.1% BSA) was added to the wells of the second group. The plates were then incubated at room temperature for 1 h and washed three times with PBS–0.05% Tween. One hundred microliters of a solution of PBS–0.1% BSA–0.05% Tween was then added to all wells except those in the first row, which received 150-μl aliquots of 1/1,000 dilutions of the sera in PBS–0.1% BSA. These samples were then titrated out with serial threefold dilutions and incubated at room temperature for 1 h. After three washes with PBS–0.05% Tween, antibodies bound to CTB and LTB were assayed by incubation with anti-mouse IgG conjugated with horseradish peroxidase (Jackson Immunoresearch). The chromogenic substrate was *o*-phenylenediamine, and titers were calculated in the same manner as for the determination of in vitro antigenicity described above.

CTB antibody absorption. Sera were absorbed with CTB-Sepharose beads in order to remove both CTB-specific antibodies and antibodies that would cross-

react with LTB. CTB-Sepharose was prepared by covalently linking CTB pentamers to cyanogen bromide-activated Sepharose (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions.

The sera were diluted 1:10 in PBS, and then 100 μl of this dilution was incubated in a microcentrifuge tube with 50 μl of Sepharose-CTB beads with intermittent shaking for 1 h at room temperature. The sera were then centrifuged at 9,000 rpm in a benchtop microcentrifuge for 2 min, and the supernatant was recovered. The procedure was repeated until the CTB titers were reduced to undetectable levels. Absorbed sera were assayed against CTB and LTB by GM1 ELISA as described above.

Toxin neutralization tests. Sera obtained after the third immunization of mice with the various CTB derivatives and LTB were tested in a Chinese hamster ovary (CHO) cell assay (6) for the ability to inhibit the toxicity of CT or LT before and after absorption with CTB-Sepharose. Unabsorbed sera were first diluted 1:30; 50-μl aliquots of the resulting sera were then serially diluted in duplicate in microtiter plates with twofold dilutions. To the first group of duplicate wells was added 0.2 ng of CT in 50 μl of Ham F12 medium containing 1% BSA; to the second group was added a solution of LT in the same medium. The plates were incubated for 1 h at room temperature, after which 100 μl of a suspension of 2×10^5 cells per ml in Ham F12 medium was added to all the wells. The incubation was allowed to proceed for 24 h at 37°C in a 10% CO_2–5% O_2 atmosphere. The cells were fixed with methanol and stained with Giemsa solution (Merck). The cells were observed for toxin-related distortion by light microscopy. The same procedure was applied to the sera after they were absorbed with CTB-Sepharose beads except that the sera were not further diluted before application to the plates. All experiments were done in duplicate.

RESULTS

Generation of hybrid CTB-LTB molecules. The three hybrid toxin genes used in this study were generated from two different CTB expression vectors. In the first, modifications were achieved by the insertion of synthetic oligonucleotides between convenient restriction sites within the *ctxB* gene, and in the second, mutations were generated by the use of specific primers in the PCR amplification of entire plasmid as previously described (21). The two resulting plasmids were pML-LCTB*tac*A and pML-LCTB*tac*B carrying modified *ctxB* genes whose sequences were confirmed by DNA sequencing and are indicated in Fig. 1. The hybrid protein LCTB*A* expressed by pML-LCTB*tac*A is identical to LTB in its first 25 amino acids and thereafter is identical to CTB. That expressed by pML-LCTB*tac*B and designated LCTB*B* is altered to correspond to LTB at positions 1, 94, and 95 but is otherwise identical to native CTB from classical O1 *V. cholerae* strain 395.

An additional hybrid protein, LCTB*H*, was also tested. This protein is a combination of the mutants LCTB*A* and LCTB*B* and thus carries the first 25 amino acids of LTB and is also altered at positions 94 and 95. It was expressed from plasmid pML-LCTB*tac*H, which was generated in the same way as pML-LCTB*tac*B except that the pML-LCTB*tac*A *ctxB* sequence was transferred to pML-CTB*tac* in order to generate a plasmid without an *Eco*RI site, and the resulting plasmid was used as the template in PCR-directed mutagenesis.

Expression of the hybrid proteins in *V. cholerae* JS1569 resulted in accumulation of the products in the growth medium, from which they could be purified by hexametaphosphate precipitation followed by HPLC.

The proteins appeared on the basis of SDS-PAGE to be similar to the control CTB and LTB proteins in both the pentameric and monomeric forms, as demonstrated by comparisons of LCTB*A* and LCTB*B* with recombinant CTB and LTB. LCTB*H* (not shown) behaved in the same manner (Fig. 2).

Antigenic analysis of CTB-LTB hybrids. The hybrid proteins were partially purified from the growth medium of *V. cholerae* carrying the respective expression vectors and subjected to a variety of different assays in order to determine their antigenic properties in comparison with native CTB and LTB.

Initially, fixed concentrations of the different proteins were titrated with different CTB- or LTB-specific antibodies in GM1 ELISA. The method will measure affinity differences for either or both the GM1 receptor and the different detecting antibodies, but we have previously shown that the different proteins bind identically to GM1 and that the differences were therefore antibody specific (results not shown). The results in Table 2 show that the profiles of the titers obtained for a range of both monoclonal antibodies and adsorbed polyclonal antisera can differ between hybrid and native proteins. The results indicate that the hybrid proteins LCTB*A* and LCTB*H* have acquired LTB-specific epitopes that can be recognized on the basis of reactions with monoclonal antibodies, notably epitopes recognized by the LTB-specific monoclonal antibody LT33:8. However, they both still react with the CTB-specific antibody CTWi and fail to react with a second LTB-specific monoclonal antibody, LT80:7. This was also demonstrated by Western blot (immunoblot) analysis (data not shown). In the case of LCTB*B*, none of the monoclonal antibodies used demonstrated any changes in the antigenicity of the hybrid molecule.

When the different proteins were assayed against a rabbit antiserum raised against LTB before and after absorption with CTB-Sepharose, it was found that both LCTB*A* and LCTB*H* gave titers similar to those of LTB, whereas LCTB*B* behaved more like CTB.

Immunizations with hybrid proteins give rise to cross-reactive antisera. Following immunization with either LTB, CTB, or the different hybrid molecules, resulting sera were analyzed for levels of overall antibodies against LTB and CTB and subsequently for levels of LTB-specific antibodies.

Figure 3A shows the levels of antibodies against CTB and LTB after the last of the three immunizations. Prior to the first immunization, there were no detectable titers against either B subunit in any of the mice tested, and pooled preimmune serum from each group of mice was used to obtain background levels for antitoxin titer determinations after immunization. In the cases of CTB and two of the hybrid proteins, the titers against CTB were slightly higher than those against LTB. In the mice immunized with LCTB*H* and LTB, the situation was reversed, with higher titers against LTB being observed.

Testing of whole sera for titers of antibodies against LTB or CTB is complicated by the presence of cross-reactive antibodies. To determine whether the differences observed in the different sera were due to changes in the levels of LTB-specific antibodies, sera taken for each group after the third immunization were pooled and absorbed with CTB-Sepharose to remove both cross-reactive and CTB-specific antibodies. They were then assayed for anti-CTB and anti-LTB titers. The results are shown in Fig. 3B. The treatment effectively removed all anti-CTB antibodies from the sera (data not shown). In mice immunized with CTB, the entire observed anti-LTB titer is due to cross-reactive antibodies, and no LTB-reactive antibodies remain after absorption. In the cases of both LCTB*A* and LCTB*B* significant anti-LTB titers remain, although these are not as high as in mice immunized with LTB. It is evident that the mutations introduced into the CTB molecule have an immunological effect giving rise to LTB-specific antibodies. It is also clear that in LCTB*A*, in which there were seven substitutions in the CTB molecule, the effect is greater than in LCTB*B*, in which there are only three, and that LCTB*H*, in turn, gave rise to higher anti-LTB titers than LCTB*A* did. Indeed, in these experiments, LCTB*H* was indistinguishable from LTB in the levels of LTB-specific antibodies obtained after immunization.

The results of intranasal immunization were essentially the same as those obtained by i.p. administration of the antigen with respect to both the overall levels of antitoxin antibodies

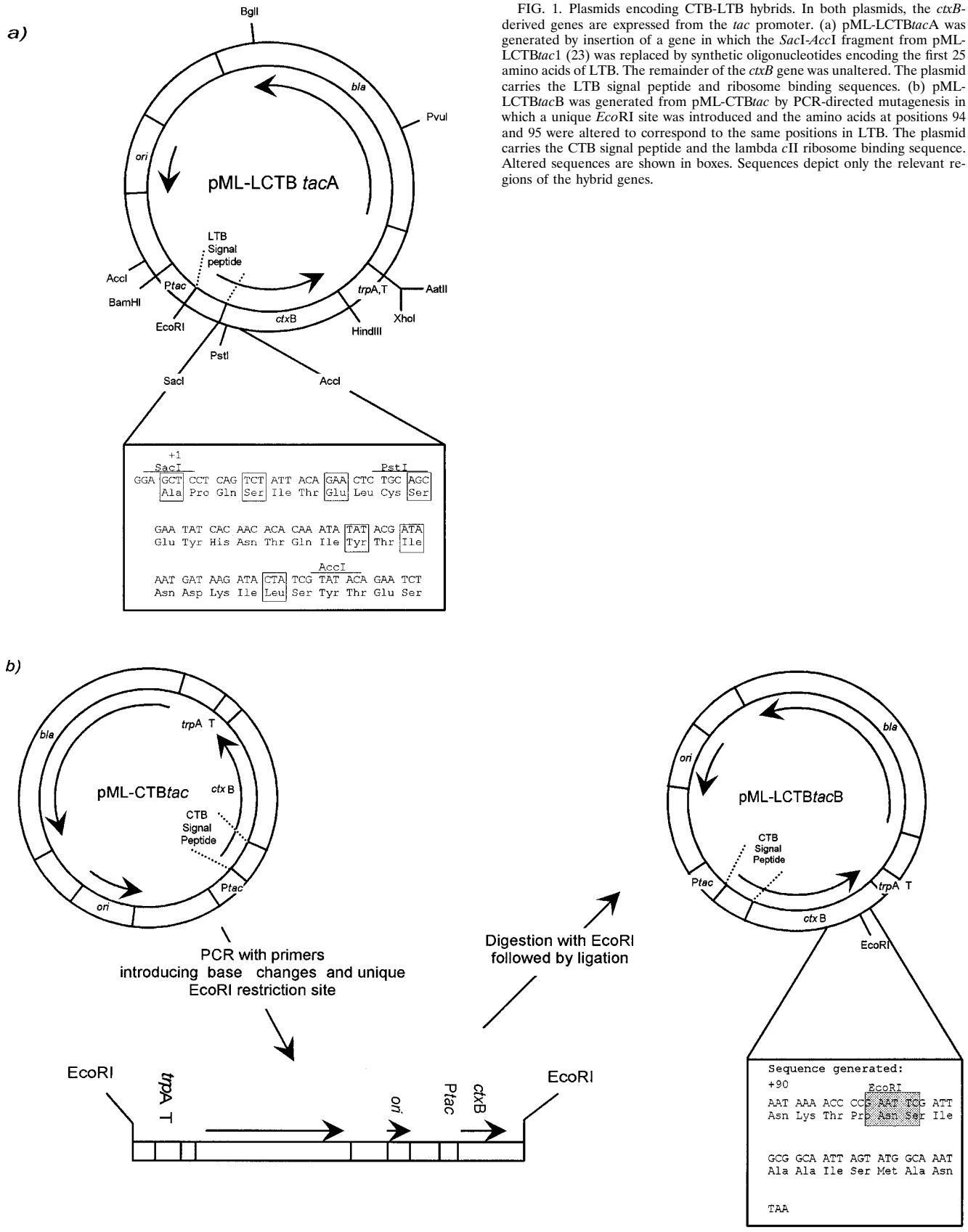

FIG. 1. Plasmids encoding CTB-LTB hybrids. In both plasmids, the *ctxB*-derived genes are expressed from the *tac* promoter. (a) pML-LCTB*tac*A was generated by insertion of a gene in which the *Sac*I-*Acc*I fragment from pML-LCTB*tac*1 (23) was replaced by synthetic oligonucleotides encoding the first 25 amino acids of LTB. The remainder of the *ctxB* gene was unaltered. The plasmid carries the LTB signal peptide and ribosome binding sequences. (b) pML-LCTB*tac*B was generated from pML-CTB*tac* by PCR-directed mutagenesis in which a unique *Eco*RI site was introduced and the amino acids at positions 94 and 95 were altered to correspond to the same positions in LTB. The plasmid carries the CTB signal peptide and the lambda *c*II ribosome binding sequence. Altered sequences are shown in boxes. Sequences depict only the relevant regions of the hybrid genes.

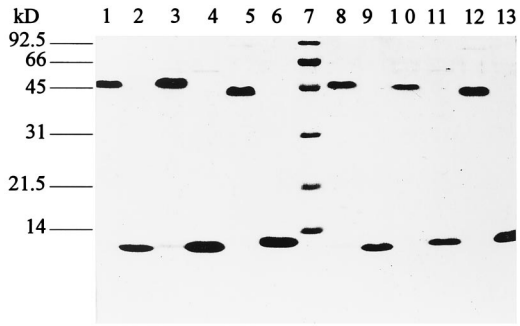

FIG. 2. SDS-polyacrylamide gel (17%) comparing 1 μg of purified CTB-LTB hybrid proteins with rCTB and rLTB before and after denaturation by boiling. Unboiled and boiled samples of each protein are arranged in pairs. Lanes: 1 and 2, rCTB (unboiled and boiled, respectively); 3 and 4, LCTB*A*; 5 and 6, rLTB; 7, low-molecular-weight protein size markers (Bio-Rad); 8 and 9, rCTB; 10 and 11, LCTB*B*; 12 and 13, rLTB.

and the levels of LTB-specific antibodies obtained with LTB and the different hybrid proteins (data not shown).

Neutralization of CT and LT by different B-subunit antisera. Pooled antisera from the different groups of mice were tested in an in vitro assay for the ability to neutralize either LT or CT. The results are shown in Table 3. Under the conditions used in the assay, CTB and LTB antisera were not significantly cross-reactive; LTB antiserum did not neutralize CT at any dilution within the range, and CTB antiserum had no effect on LT. In contrast, antisera to the hybrid molecules had significant neutralizing activity against both CT and LT. Even after complete absorption of the CT-neutralizing activity of the antisera with CTB, there was still significant LT-neutralizing activity. These results indicate that the substitutions made within the CTB molecule introduce neutralizing LTB epitopes while at the same time retaining CT neutralization epitopes. It is significant that there were only slight differences between the levels of neutralizing activity demonstrated against LT by antiserum from mice immunized with the different hybrid proteins regardless of the levels of LTB-specific antibodies observed by GM1 ELISA. This finding suggests that the amino acids at positions 94 and 95 of the B-subunit structure and residues 1 to 25 each form part of independent, powerful neutralizing epitopes. However, the effect of combining them clearly does not result in a molecule that is able to more effectively neutralize LT.

TABLE 2. Generation of LTB-specific epitopes with retention of CTB epitopes in hybrid proteins[a]

Antibody tested	Log_{10} antibody titer to:				
	rCTB	LCTB*A*	LCTB*B*	LCTB*H*	LTB
Monoclonal antibodies					
LT39	4.3	4.4	4.1	4.3	4.1
LT33:8	0	4.3	0	3.7	4.2
LT80:7	0	0	0	0	3.9
CTWi	3.4	2.8	3.0	3.15	0
Polyclonal antisera					
R953	4.4	4.7	4.3	4.8	4.9
R953abs	2.2	4.4	2.5	4.4	4.7

[a] Antibody titers were determined by a GM1 ELISA procedure (see Materials and Methods). LT39 is a cross-reactive monoclonal antibody reacting similarly with both LTB and CTB. LT33:8 and LT80:7 are LTB-specific monoclonal antibodies, and CTWi is a CTB-specific monoclonal antibody. R953 is a rabbit polyclonal hyperimmune serum raised against LTB, and R953abs is the same serum after absorption with CTB. Estimated titer differences exceeding log_{10} = 0.5 (i.e., threefold) are considered significant.

TABLE 3. Increased LT-neutralizing activities of antisera generated by hybrid proteins

Immunization with:	Neutralization titer[a]			
	Nonabsorbed serum		Absorbed serum	
	CT	LT	CT	LT
CTB	240	<30	<30	<30
LCTB*A*	120	60	<30	60
LCTB*B*	120	60	<30	60
LCTB*H*	60	30	<30	60
LTB	<30	240	<30	120

[a] Neutralization of LT and CT by sera obtained after three immunizations of mice with CTB, LTB, or either of the three LTB-CTB hybrids was determined in a CHO cell assay (see Materials and Methods). The sera were also extensively absorbed with CTB-Sepharose and assayed by the same procedure. Experiments were conducted such that the starting concentrations in the first wells were equivalent in experiments with sera before and after absorption with CTB-Sepharose. To achieve this, unabsorbed sera were diluted 1:30 in the first well, whereas following absorption they were used undiluted. Absorbed and unabsorbed sera were serially diluted twofold. Displayed titers indicate the maximum dilutions at which sera protected cells from distortion and indicate the range obtained from titrations performed in two separate duplicated experiments.

DISCUSSION

This report describes the synthesis and analysis of three hybrid B-subunit toxin molecules essentially composed of CTB in which mutations have altered specific amino acid residues to resemble the corresponding positions in human LTB. The two regions chosen for alteration were designed to address the question of whether possible targets for the immune system encompass both structural and functional features of the hybrids and the relative significance of each in mounting a protective response.

All of the proteins were found to be expressed at high levels and to retain the essential characteristics of both CTB and LTB. They all assembled into pentamers and bound to GM1. In addition, the three molecules reacted similarly to the wild-type CTB and LTB with the cross-reacting monoclonal antibody LT39 and also reacted strongly with polyclonal antisera to either CTB or LTB. In reactions with other LTB- or CTB-specific monoclonal antibodies, however, we could make distinctions between the different proteins that suggested that the novel proteins did indeed carry epitopes specific to each of the wild-type proteins on the same molecule. This observation was further illustrated by both i.p. and mucosal immunization experiments, which demonstrated an increased cross-reactivity of sera obtained from animals immunized with the hybrid proteins. It was demonstrated in the CHO cell toxin neutralization assay that antisera to the hybrid molecules neutralized LT more effectively than did antiserum to CTB alone and that this activity could not be abolished by absorption with CTB.

The hybrid molecules used in the present work were generated to illustrate the effects of mutations at distinct regions of the CTB molecule. In the first construct, the cluster of differences at the amino terminus of CTB were altered, whereas in the second, two residues were changed at a locus close to the carboxy terminus thought to be involved in the substrate specificity of receptor binding. The mutation Thr→Ala at position 1 of LCTB*B* was not investigated in this study. The recombinant CTB used for controls was produced from an expression system described previously (13) and also carried this muta-

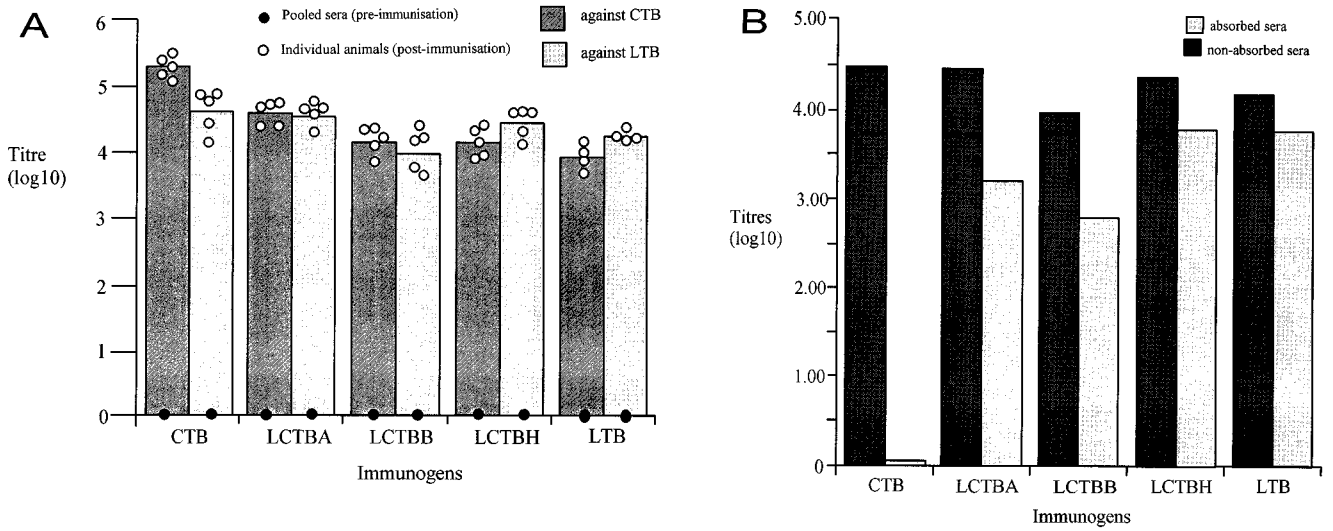

FIG. 3. (A) Antitoxin antibody responses to immunization (three times i.p.) with either rCTB, rLTB, or the CTB-LTB hybrid protein LCTB*A*, LCTB*B*, or LCTB*H*. Geometric mean titers of IgG antitoxin, determined by GM1 ELISA, against CTB and LTB are shown together with the titers obtained for the five individual mice in each group (open circles); the closed circles indicate the undetectable levels of antibodies found with pooled preimmune sera from each group. Note that one of the mice in the LTB group died during the experiment. (B) Effect of absorption with CTB on titers of CTB- and LTB-reactive antibodies in sera of mice obtained after three immunizations with either rCTB, rLTB, or the CTB-LTB hybrid protein LCTB*A*, LCTB*B*, or LCTB*H*. The graph shows GM1 ELISA titers obtained with the sera on LTB-coated plates (LTB-reactive antibody titers) before and after absorption with CTB-Sepharose immunosorbent beads. In similar experiments using CTB-coated plates, CTB titers in absorbed sera were undetectable (data not shown).

tion, which we have previously shown to have no immunological significance (12, 13).

From the results, it is clear that the more extensive alteration at the amino terminus of CTB gives rise to more LTB-specific antibodies (as defined by GM1 ELISA antibody titers that are not absorbed by CTB-Sepharose) compared with the two-residue change at positions 94 and 95 of the mature protein. Nonetheless, the latter change also gave significant anti-LTB GM1 ELISA titers, despite the fact that the molecule was indistinguishable from CTB, using the panel of individual monoclonal antibodies used in initial antigenic analyses.

The third hybrid protein, which combined the mutations generated in the other two, appeared to behave essentially in the same way as LTB, and anti-LTB titers were higher than anti-CTB titers in the animals immunized with this construct. In addition, the residual titers against LTB after absorption with CTB-Sepharose were also similar to those observed with group immunized with LTB and significantly higher than those obtained with the other two hybrids.

It therefore appears that amino acid changes in CTB at 9 of the 17 positions that differ from human LTB convert the molecule to one that carries the majority of the LTB-specific epitopes and that the regions of the molecule that are important in this regard can be localized to the first 25 amino acids of the molecule and to residues 94 and 95. These findings may reflect the cluster of 7 of the 17 differences between CTB and human LTB found in the first 25 residues of the molecules and the functional significance of position 95 in the determination of receptor binding specificities that also differ so markedly between the two molecules.

Also of considerable interest, although somewhat unexpected, was the finding that neutralization of LT by antisera to the mutant B subunits in the CHO cell assay were similar and uniformly greater than that of antiserum raised against CTB. Although the assay is relatively insensitive, the results suggest that despite the apparent lower titer of LTB-specific antibodies generated by the LCTB*B* hybrid and the relatively higher titers generated by LCTB*H* than by LCTB*A*, the antibodies have equivalent neutralizing effects. It is further evident that there is more than one neutralizing epitope on the B-subunit molecule, since none of the mutants lose the ability to neutralize CT despite the likelihood that some CTB-specific neutralizing epitopes were lost as a result of the substitutions.

The cross-reactivity of the mutant CTB molecules in the toxin neutralization assay could be attributed to the specific changes generated, since these gave rise to LTB-specific antibodies unaffected by immunosorbent removal of CTB-specific as well as all cross-reactive antibodies that resulted in the complete loss of CT-neutralizing activity from all samples in the CHO cell assay. A portion of the LT-neutralizing activity, however, was retained.

The combined results for LCTB*H* indicate that even in this molecule, there remain CTB-specific neutralizing epitopes that explain the continued ability of LCTB*H* antisera to neutralize CT in vitro. Furthermore, the neutralizing effects of the changes in LCTB*A* and LCTB*B* are not apparently additive and perhaps indicate that epitopes in one region of the molecule predominate when both sets of alterations are present. This conclusion is supported by the result obtained when the different molecules were tested for antigenicity by using rabbit antiserum raised against LTB before and after absorption with CTB-Sepharose. It could be seen that LCTB*A* gave higher titers than LCTB*B* with the absorbed antiserum, suggesting that the first 25 residues of LTB carry dominant specific epitopes and that it was these that contributed to the result observed with LCTB*H* when the same antiserum was used. Thus, in the mice immunized with LCTB*B* in which only the alterations at positions 94 and 95 are present, this is a dominant LTB-specific epitope, whereas in the presence of the alterations in LCTB*A*, the LTB-specific epitopes present in the latter are dominant.

From these investigations, it is clear that hybrid molecules containing both CTB- and LTB-specific neutralizing epitopes can be generated, although the contribution of these epitopes

to neutralization of toxins by resulting antisera appears to be more complex than previously thought. In our further work, we plan to systematically analyze various additional hybrid molecules carrying single and combined specific LTB alterations in the CTB protein with regard to their immunological properties and potential for use in vaccines. The recently described overexpression system for CTB allows these proteins to be produced in large quantities. Thus, such molecules could potentially be incorporated either into separate cholera and ETEC vaccines or included in a single broad-spectrum vaccine designed to give significant protection against both types of diarrheal disease.

ACKNOWLEDGMENTS

We thank G. Wallerström for assistance with the CHO cell assay.

This work was funded by a grant from the Swedish Biotechnology Research Council (project 92-864) and the Swedish Medical Research Council (grant 16X3382 and 16X9503).

REFERENCES

1. **Åhrén, C., C. Wenneråas, J. Holmgren and A.-M. Svennerholm.** 1993. Intestinal antibody response after oral immunisation with a prototype B subunit-CFA enterotoxigenic *Escherichia coli* vaccine. Vaccine **11:**929–935.
2. **Black, R. E.** 1986. The epidemiology of cholera and enterotoxigenic E. coli diarrheal diseases, p. 23–32. *In* J. Holmgren, A. Lindberg, and R. Mölby (ed.), Development of vaccines and drugs against diarrhea. Proceedings of the 11th Nobel Conference. Studentlitteratur, Lund, Sweden.
3. **Clemens, J., D. Sack, J. R. J. Harris, J. Chakraborty, P. K. Neogy, B. Stanton, N. Huda, M. U. Khan, B. A. Kay, M. R. Khan, M. Ansaruzzaman, M. Yunus, M. R. Rao, A.-M. Svennerholm, and J. Holmgren.** 1988. Cross-protection by B subunit-whole cell cholera vaccine against diarrhea associated with heat-labile toxin-producing enterotoxigenic *E. coli*: results of a large field trial. J. Infect. Dis. **158:**372–377.
4. **Clemens, J., D. Sack, J. R. J. Harris, F. van Loon, J. Chakraborty, F. Ahmed, M. R. Rao, M. R. Khan, M. Yunus, N. Huda, B. Stanton, B. A. Kay, S. Walter, R. Eeckels, A.-M. Svennerholm, and J. Holmgren.** 1988. Field trials of oral cholera vaccines in Bangladesh: results from three-year follow-up. Lancet **i:**270–273.
5. **Domenighini, M., M. Pizza, M. G. Jobling, R. K. Holmes, and R. Rappuoli.** 1995. Identification of errors among the database sequence entries and comparison of correct amino acid sequences for the heat-labile enterotoxin of *Escherichia coli* and *Vibrio cholerae*. Mol. Microbiol. **15:**1165–1167.
6. **Guerrant, R. L., L. L. Brunton, R. C. Schnaitman, L. I. Rebhun, and A. G. Gliman.** 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive in vitro assay for enterotoxins of *Vibrio cholerae* and *Escherichia coli*. Infect. Immun. **10:**320–327.
7. **Holmgren, J.** 1981. Actions of cholera toxin and the prevention and treatment of cholera. Nature (London) **292:**413–417.

7a. **Holmgren, J., et al.** Unpublished data.

8. **Holmgren, J., P. Fredman, M. Lindblad, A.-M. Svennerholm, and L. Svennerholm.** 1982. Rabbit intestinal glycoprotein receptor for *Escherichia coli* heat-labile enterotoxin lacking affinity for cholera toxin. Infect. Immun. **38:** 424–433.
9. **Holmgren, J., A.-M. Svennerholm, M. Jertborn, J. Clemens, D. A. Sack, R. Salenstedt, and H. Wigzell.** 1992. An oral B subunit: whole cell vaccine against cholera. Vaccine **10:**911–914.
10. **Institute of Medicine.** 1986. New vaccine development, establishing priorities, vol. II. Diseases of importance in developing countries. National Academy Press, Washington, D.C.
11. **Kaper, J. B., H. Lockman, M. M. Baldini, and M. M. Levine.** 1984. Recombinant nontoxigenic *Vibrio cholerae* strains as attenuated cholera vaccine candidates. Nature (London) **308:**655–658.
12. **Lebens, M., and J. Holmgren.** Unpublished data.
13. **Lebens, M., S. Johansson, J. Osek, M. Lindblad, and J. Holmgren.** 1993. Overexpression of recombinant cholera toxin B subunit for use in oral vaccines. Bio/Technology **11:**1574–1578.
14. **Leong, J., A. C. Vinal, and W. S. Dallas.** 1985. Nucleotide sequence comparison between heat-labile toxin B subunit cistrons from *Escherichia coli* of human and porcine origin. Infect. Immun. **48:**73–78.
15. **Merrit, E. A., S. Sarfaty, F. van den Akker, C. L'Hoir, J. A. Martial, and W. G. J. Hol.** 1994. Crystal structure of cholera toxin B pentamer bound to receptor GM1 pentasaccharide. Protein Sci. **3:**166–175.
16. **Peltola, H., A. Siitonen, H. Kyrönseppä, I. Simula, L. Mattila, P. Oksanen, M. J. Kataja, and M. Cadoz.** 1991. Prevention of travellers' diarrhoea by oral B subunit/whole cell cholera vaccine. Lancet **338:**1285–1289.
17. **Quiding, M., I. Nordström, A. Kilander, G. Andersson, L.-Å. Hanson, J. Holmgren, and C. Czerkinsky.** 1991. Intestinal immune responses in humans. Oral cholera vaccination induces strong intestinal antibody responses and interferon-γ production and invokes immunological memory. J. Clin. Invest. **88:**143–148.
18. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Sanchez, J., and J. Holmgren.** 1989. Recombinant system for overexpression of cholera toxin B subunit in *Vibrio cholerae* as a basis for vaccine development. Proc. Natl. Acad. Sci. USA **86:**481–485.
20. **Sandkvist, M., T. Hirst, and M. Bagdasarian.** 1987. Alterations at the carboxyl terminus change assembly and secretion properties of the B subunit of *Escherichia coli* heat-labile enterotoxin. J. Bacteriol. **169:**4570–4576.
21. **Schödel, F., D. R. Milich, and H. Will.** 1991. Hybrid hepatitis B virus core/pre-S particles expressed in attenuated *Salmonellae* for oral immunisation, p. 319–325. *In* F. Brown, R. M. Chanock, H. S. Ginsberg, and R. A. Lerner (ed.), Vaccines 91. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Sixma, T. K., K. H. Kalk, B. A. M. van Zanten, Z. Dauter, J. Kingma, B. Witholt, and W. G. J. Hol.** 1993. Refined structure of *Escherichia coli* heat-labile enterotoxin, a close relative of cholera toxin. J. Mol. Biol. **230:**890–918.
23. **Sixma, T. K., S. E. Pronk, K. H. Kalk, E. S. Wartner, B. A. M. van Zanten, B. Witholt, and W. G. J. Hol.** 1991. Crystal structure of a cholera toxin-related heat-labile enterotoxin from *E. coli*. Nature (London) **351:**371–378.
24. **Spangler, B. D.** 1992. Structure and function of cholera toxin and the related heat-labile enterotoxin of *Escherichia coli*. Microbiol. Rev. **56:**622–647.
25. **Svennerholm, A.-M., C. Åhren, C. Wenneråas, and J. Holmgren.** 1991. Development of an oral vaccine against enterotoxigenic *E. coli* diarrhea, p. 287–294. *In* T. Wadström, H. Mekele, A.-M. Svennerholm, and H. Wolf-Watz (ed.), Molecular pathogenesis of gastrointestinal infections. Plenum Press, London.
26. **Svennerholm, A.-M., L. Gothefors, D. A. Sack, P. K. Bardhan, and J. Holmgren.** 1984. Local and systemic antibody responses and immunological memory in humans after immunization with cholera toxin B subunit by different routes. Bull. W.H.O. **62:**909–918.
27. **Svennerholm, A.-M., and J. Holmgren.** 1976. Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. Infect. immun. **13:**735–740.
28. **Svennerholm, A.-M., and J. Holmgren.** 1978. Identification of Escherichia coli heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM1 ELISA) procedure. Curr. Microbiol. **1:**19–23.
29. **Svennerholm, A.-M., J. Holmgren, R. Black, M. Levine, and M. M. Merson.** 1983. Serologic differentiation between antitoxic responses to infection with *Vibrio cholerae* and enterotoxin-producing *Escherichia coli*. J. Infect. Dis. **147:** 514–522.
30. **Svennerholm, A.-M., M. Jertborn, L. Gothefors, A. M. M. M. Karim, D. A. Sack, and J. Holmgren.** 1984. Mucosal antitoxic and antibacterial immunity after cholera disease and after immunization with a combined B subunit-whole cell vaccine. J. Infect. Dis. **149:**884–893.
31. **Svennerholm, A.-M., D. A. Sack, J. Holmgren, and P. K. Bardhan.** 1982. Intestinal antibody responses after immunization with cholera toxin B subunit. Lancet **i:**305–308.
32. **Svennerholm, A.-M., M. Wickström, M. Lindblad, and J. Holmgren.** 1986. Monoclonal antibodies to *Escherichia coli* heat-labile enterotoxins: neutralising activity and differentiation of human and porcine LTs and cholera toxin. Med. Biol. **64:**23–30.

Editor: P. J. Sansonetti