INFECTION AND IMMUNITY, Jan. 1993, p. 48–55
0019-9567/93/010048-08$02.00/0

Vol. 61, No. 1

Comparative Effectiveness of the Cholera Toxin B Subunit and Alkaline Phosphatase as Carriers for Oral Vaccines

MARK T. DERTZBAUGH†* AND CHARLES O. ELSON

Division of Gastroenterology, School of Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294

Received 7 August 1992/Accepted 15 October 1992

The purpose of this study was to determine whether the B subunit of cholera toxin (CtxB) has adjuvant activity over and above serving as a carrier protein for orally administered vaccines. An oligonucleotide that encodes an antigenic determinant (GtfB.1) from the glucosyltransferase B gene (*gtfB*) of *Streptococcus mutans* was genetically fused to the 5′ terminus of either the CtxB gene (*ctxB*) or the *Escherichia coli* alkaline phosphatase gene (*phoA*). The resulting chimeric proteins were expressed in a *phoA* mutant strain of *E. coli* and then purified. The antigenicities of the proteins were confirmed by immunoblotting analysis using antisera specific for GtfB, CtxB, or PhoA. An equimolar amount of peptide on each carrier was administered by gastric intubation to mice three times at 10-day intervals. Antibody titers to the peptide, CtxB, and PhoA (in the serum, intestine, vagina, saliva, and bronchus) were determined by enzyme immunoassay. Antibody to the peptide was detected only in the sera of mice immunized with the peptide fused to CtxB. No antipeptide antibody was detected in mice immunized with the peptide fused to PhoA. The lack of detectable levels of antipeptide antibody in intestinal lavage fluid was attributed to dilution of the sample beyond the sensitivity of the assay. This was confirmed by cultivation of Peyer's patch and mesenteric lymph node tissue from mice orally immunized with the GtfB.1::CtxB chimera. Using this method, antipeptide antibody was detected in the culture fluid. We conclude that CtxB possesses unique properties that allow it to act as more than a simple carrier protein.

In the past several years, there has been an increased awareness of the importance of mucosal immunity in host defense against colonization and invasion by pathogenic microorganisms. This has highlighted the need for the development of effective oral vaccines. Unfortunately, most nonreplicating antigens are poor immunogens when administered orally (4), and this weakness has limited the number of vaccines targeted for delivery to the mucosal immune system.

In addition to being a potent mucosal immunogen (11, 30), cholera toxin (Ctx) has been found to act as an adjuvant for mucosally administered nonreplicating antigens (12, 21, 23). Ctx is composed of two subunits: a toxigenic A subunit (CtxA) and a B subunit (CtxB) that mediates binding of CtxA to the surface of eukaryotic cells via its interaction with the monosialoganglioside GM_1 (16). Because of the toxicity of Ctx, several investigators have used simple coadministration (23, 33) or chemical conjugation to another antigen (7, 26) to explore the potential of the nontoxic CtxB to act as a mucosal adjuvant. CtxB has been reported to act as an adjuvant in some cases (26, 33) but not in others (7, 21, 23). The reason for this discrepancy is not clear, but the discrepancy may depend on several factors such as the antigen used, the method of conjugation, the dosage, the route of immunization, and whether the CtxB preparations used were contaminated with trace amounts of holotoxin.

In order to address some of these issues, we constructed a set of fusion proteins composed of the same antigenic peptide sequence genetically fused to CtxB or alkaline phosphatase (PhoA) of *Escherichia coli*. The peptide sequence used was derived from the *gtfB* gene of *Streptococcus mutans*, and the fusion of this sequence to CtxB has been described elsewhere (10). Furthermore, the fusion proteins were constructed so that they retained their respective biological properties, such as GM_1 binding for CtxB and enzymatic activity for PhoA.

Our hypothesis was the following: if CtxB was acting as more than a simple carrier protein for oral vaccination, then feeding the GtfB::CtxB fusion protein to mice should result in a greater antibody response to the peptide than is achieved by feeding an equimolar amount of the same peptide fused to PhoA.

MATERIALS AND METHODS

Bacteria, plasmids, and protein. All bacteria and plasmids used in this study are listed in Table 1. Plasmid DNA was prepared by sodium dodecyl sulfate (SDS)–high-salt lysis (17) followed by dye–buoyant-density equilibrium centrifugation (34). For production of protein, plasmids were transformed into the *phoA* mutant *E. coli* strain AW1043 by the $CaCl_2$-heat shock method (28). Growth of this strain required selection on 25 μg of kanamycin per ml in order to maintain the transposon insertion used to inactivate the chromosomal copy of *phoA*. Bacteria were grown at 37°C in M-9 medium (27) containing 50 μg of ampicillin per ml and supplemented with 10 g of Casamino Acids (Difco, Ann Arbor, Mich.) per liter, 20 μg each of leucine and proline per ml, and 2 μg of thiamine per ml. For expression of enzymatically active PhoA, the medium described above was supplemented with 5 μM ZnCl. For fermentor-scale growth, bacteria were grown to an optical density of 0.4 at 660 nm before the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma Chemical Co., St. Louis, Mo.) to a concentration of 1 mM to induce expression of the chimeric proteins.

* Corresponding author.
† Present address: U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD 21702-5011.

TABLE 1. Bacterial strains and plasmids

E. coli strain	Plasmid	Comments	Reference
HB101	None	F⁻*hsdS20*(r_B^- m_B^-) *recA1 leuB6 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	2
AW1043	None	F⁻ *lac galU galK* (*leu-ara*) *phoA*-E15 *proC*::Tn5	18
P22	pINIII*ompA2*	*ompA* secretion-expression vector	15
IN166	pIN166	*phoA* expression vector	14
V1599	pVA1599	*gtfB.1*::*ctxB* gene fusion	10
V1782	pVA1782	*gtfB.1*::*ctxB* expression vector	10
MTD12	pMTD12	pINIII*ompA2* with deletion of *Nde*I site	This study
MTD14	pMTD14	*gtfB.1*::*ctxB* expression vector	This study
MTD15	pMTD15	*gtfB.1*::*phoA* expression vector	This study
MTD20	pMTD15	pMTD15 expressed in host AW1043	This study
MTD46	pVA1782	pVA1782 expressed in host AW1043	This study

Genetic construction. The construction of plasmid pMTD15, which encodes the *gtfB.1*::*phoA* gene fusion, is described in Fig. 1. Primers used for the modification and amplification of *phoA* were synthesized on an Applied Biosystems 380A synthesizer (DNA Core Facility, University of Alabama at Birmingham [UAB]). Plasmid pIN166 was digested with *Hin*dIII, and the 2.5-kb fragment encoding *phoA* was isolated. This fragment was used as template, and 10 ng/μl was mixed together with 70 ng of each primer per μl. The reaction mixture was prepared by using the Geneamp amplification kit (Perkin-Elmer Cetus, Norwalk, Conn.) and then subjected to 25 cycles of amplification (melting temperature, 94°C; annealing temperature, 55°C; extension temperature, 72°C). The polymerase chain reaction (PCR)-amplified product was treated with the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories, Gaithersburg, Md.) in order to ensure complete extension prior to digestion with *Nde*I and *Bam*HI. The amplified product was inserted into pMTD14 by using T4 ligase (Bethesda Research Laboratories) to make pMTD15. The initial selection of clones expressing the GtfB.1::PhoA fusion protein was done with *E. coli* HB101 (2) as the host. Colonies were selected on the basis of their enzymatic activities on M-9 indicator plates containing 1 mM IPTG, 20 mg of 5-bromo-4-chloro-indolyl phosphate (BCIP) (Sigma) per liter, and 5 μM ZnCl.

Protein purification. The GtfB.1::CtxB fusion protein from MTD46 was isolated from the periplasm of *E. coli* by osmotic shock (10) and then purified by acidic precipitation and ion-exchange chromatography as described elsewhere (8). The GtfB.1::PhoA fusion protein from MTD20 was isolated from the periplasm by osmotic shock, and then the shock fluid was concentrated with 70% saturated NH_4SO_4. The protein was suspended and dialyzed against 10 mM Tris (pH 8.3)–10 mM MgCl–5 μM ZnCl. Most of the contaminating protein was removed by heat precipitation (6) at 70°C for 10 min followed by chilling on ice for 1 h. As a final step, the PhoA fusion protein was eluted from a DEAE-Sepharose ion-exchange column (Pharmacia, Piscataway, N.J.). The protein was suspended in 50 mM imidazole (pH 7.0) and was eluted from the column by using a linear NaCl gradient of 0 to 250 mM.

Protein analysis. For N-terminal sequence analysis, the purified proteins were reduced with 2-mercaptoethanol and then alkylated with iodoacetic acid (10) prior to sequencing (Peptide Core Facility, UAB Cancer Center). Culture supernatant from *Streptococcus mutans* GS-5 enriched for the native GtfB protein was kindly provided by S. Michalek (UAB). Purified CtxB was purchased from List Biological Laboratories (Campbell, Calif.). PhoA protein was purchased from Sigma. Protein samples were boiled for 5 min in sample buffer containing 1% SDS and 0.2 M 2-mercaptoethanol. Samples were separated by discontinuous polyacrylamide gel electrophoresis (PAGE) on an 8 to 25% gradient gel (20). For estimation of protein size, gels were stained with Coomassie blue to visualize the proteins and then compared with molecular weight markers (Bethesda Research Laboratories). For immunoblotting analysis, the proteins were transferred from the gel to nitrocellulose sheets by using a semidry electroblotter (Pharmacia). The blots were blocked in Tris-buffered saline (TBS; 50 mM Tris [pH 7.5], 140 mM NaCl) containing 1% bovine serum albumin (BSA; Sigma) prior to incubation in antiserum overnight. Polyclonal anti-CtxB was purchased from Calbiochem (La Jolla, Calif.), and monoclonal antibody to PhoA was kindly provided by J. Chlebowski, Virginia Commonwealth University, Richmond. Polyclonal anti-GtfB was a gift from H. Kuramitsu, University of Southwest Texas Medical Center, San Antonio. The blots were washed three times in TBS–0.02% Tween 20 (TTBS) and then incubated in TBS–1% BSA containing the appropriate enzyme-conjugated second antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). After three washes in TTBS, immunoreactive bands were visualized by using BCIP-nitroblue tetrazolium substrate solution (Kirkegaard & Perry).

Immunizations. Female CB6F1 mice, 4 to 6 weeks old (Jackson Laboratories, Bar Harbor, Maine), were provided food and water ad libitum. For intraperitoneal (i.p.) injection, 10 μg of protein was suspended in 20% Maalox and administered in a volume of 0.1 ml. For immunization per os (p.o.), protein was suspended in 0.2 M Na_2HCO_3 and fed in a volume of 0.5 ml by using a curved intragastric feeding needle. Mice were orally immunized on days 0, 10, and 20. Samples of serum and mucosal secretions were collected on day 27, and then the mice were challenged i.p. on days 30 and 40. On day 47, the mice were assayed for serum antibody.

Sample collection. In order to increase the volume of material available for assay, samples from identically immunized groups of mice were pooled (five mice per group). For collection of serum, mice were bled with heparinized capillary tubes at the retro-orbital plexus while under metafane anesthesia. Bronchial washings were collected as described by Nedrud et al. (29). For collection of saliva, mice were injected i.p. with 0.2 ml of a 10-mg/ml sterile solution of pilocarpine (Sigma). The mice began to salivate after approximately 2 min, and the saliva was collected by using a capillary tube. Intestinal lavage was performed by the method of Elson et al. (13). Briefly, the mice were adminis-

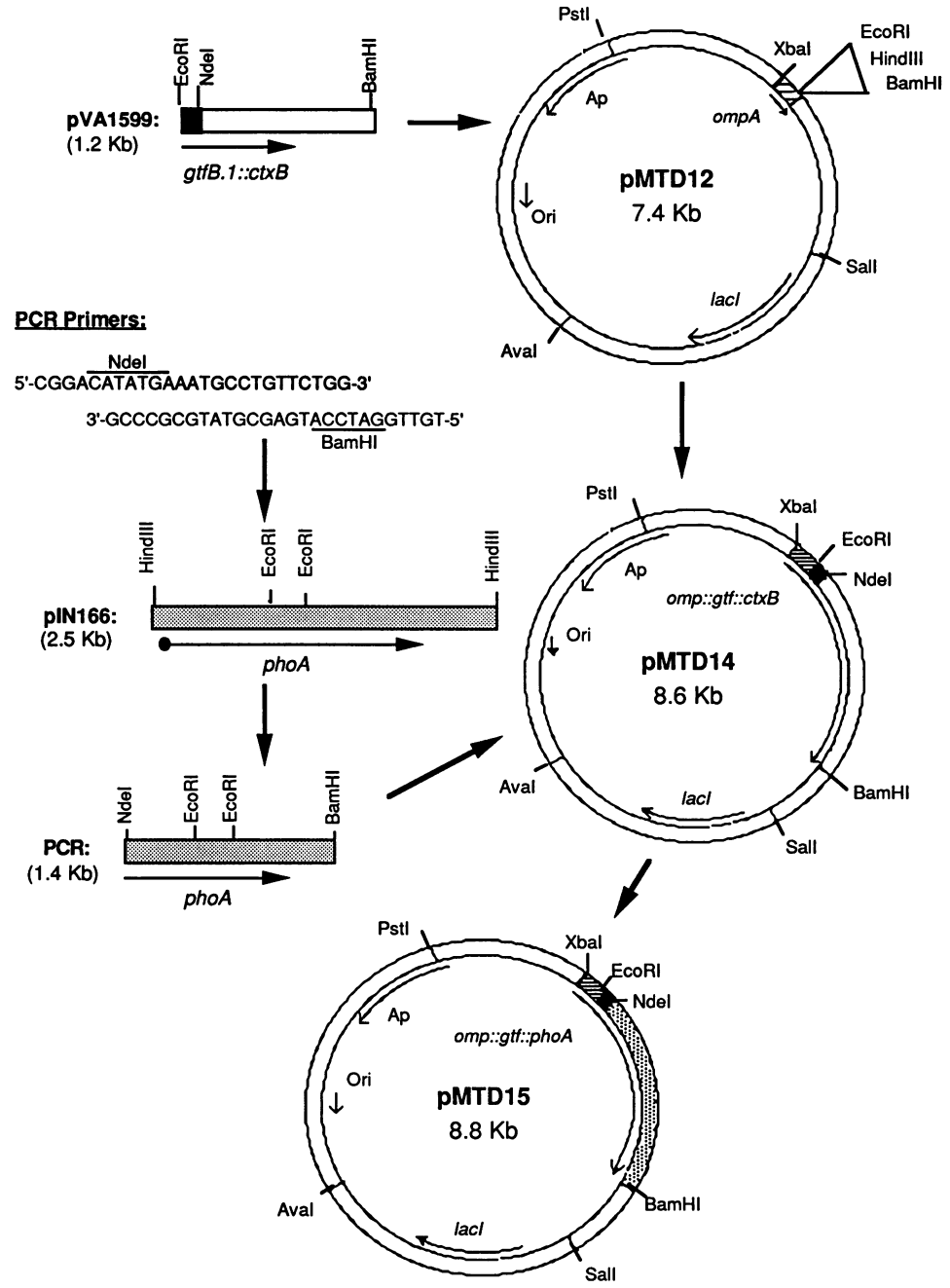

FIG. 1. Construction of the *gtfB.1*::*phoA* expression vector. A 1.2-kb *Eco*RI-*Bam*HI fragment from plasmid pVA1599, which carries *gtfB.1*::*ctxB*, was directionally inserted into pMTD12. This plasmid was derived from the secretion vector pINIII*ompA2* by deletion of an *Nde*I site. Expression of the *gtfB.1*::*ctxB* gene was inducible in pMTD14. Plasmid pIN166 was digested with *Hin*dIII, and a 2.5-kb fragment encoding the *phoA* gene was used as the template for amplification by PCR. In order to translationally fuse *phoA* to *gtfB.1*, an *Nde*I site was encoded in the sequence of the primers used for amplification. The resulting 1.4-kb PCR-modified product, encoding *phoA*, was used to replace the ~1.2-kb *Nde*I-*Bam*HI fragment of pMTD14, which carries *ctxB*, to make pMTD15. Ap, ampicillin; Ori, origin of replication.

tered 0.5 ml of Colyte solution fortified with polyethylene glycol 3300 four times at 15-min intervals with an intragastric feeding needle. Thirty minutes after the last dose, the mice were injected i.p. with 0.2 ml of a 10-mg/ml solution of pilocarpine. The intestinal contents were collected over a period of 20 min and then processed for antibody assay. Vaginal secretions were collected by using a pipettor to douche the mice with 0.1 ml of phosphate-buffered saline, which was then aspirated back into the pipette tip and used for determination of antibody levels.

Fragment cultures. Fragment cultures of Peyer's patches (PP) and mesenteric lymph nodes (MLN) were prepared by an adaptation of the method of Logan et al. (22). Mice were immunized p.o. with 69 nmol of peptide, which corresponds to either 1 mg of the GtfB.1::CtxB chimera or 3.5 mg of the GtfB.1::PhoA chimera. Five mice were used for each group.

Seven days after secondary immunization, tissues from identically immunized mice were aseptically removed and then pooled in dishes containing complete Dulbecco's minimal essential medium (10% fetal calf serum, 1% L-glutamine, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid], 2,000 U of penicillin per ml, and 20 μg of streptomycin per ml). The tissue was teased apart and then incubated in six-well tissue clusters (Falcon, Lincoln Park, N.J.) in complete Dulbecco's minimal essential medium for 5 days at 37°C in a humidified atmosphere of 7.5% CO_2 prior to assay for specific antibody.

Enzyme immunoassays. Samples were assayed for antibody levels by enzyme-linked immunosorbent assays (ELISA). Antibody samples were quantitated for their antigen specificities and isotype distributions. Polyvinyl assay plates (Falcon) were coated with 200 ng of CtxB (List) per ml, 200 ng of PhoA (Sigma) per ml, or synthetic GtfB.1 peptide conjugated to ovalbumin (Sigma). The GtfB.1 peptide was synthesized with a cysteine at its C terminus (UAB Peptide Core Facility) and conjugated to ovalbumin by using the heterobifunctional cross-linking reagent *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (Pharmacia). The optimal dilution for use as coating antigen in an ELISA was determined empirically. As a control, plates were also coated with ovalbumin alone. The plates were washed with TTBS and then blocked with TBS–1% BSA for 1 h. Samples were serially diluted in the wells of the plates and then incubated overnight at 4°C. After washing, the wells were incubated for 4 h with isotype-specific, affinity-purified horseradish peroxidase-conjugated second antibody (Kirkegaard & Perry). The plates were washed and then developed in substrate buffer (0.1 M sodium acetate, pH 6.0) containing 0.1 mg of tetramethylbenzidine (Sigma) per ml. The reaction was stopped by the addition of an equal volume of 1 M H_2SO_4, and the A_{450} of each well was determined spectrophotometrically. Specific antibody levels were determined by endpoint dilution. Titer was defined as the reciprocal of the highest endpoint dilution of sample with an A_{450} >0.050 above the negative control. The concentration of total immunoglobulin A (IgA) or IgG in each sample was determined in a separate ELISA using known concentrations of myeloma proteins such as MOPC315 (Sigma) for IgA and RPC5 (Sigma) for IgG as standards. Each pooled sample was assayed in triplicate, and the mean and standard deviation (if any) were determined.

RESULTS

Construction of the *gtfB.1* fusion proteins. The construction and characterization of pVA1782, which expresses the *gtfB.1*::*ctxB* gene fusion, has been described elsewhere (10). The construction of plasmid pMTD15, which carries the *gtfB.1*::*phoA* gene fusion, is described in Fig. 1. Plasmid pMTD14 was derived from pINIII*ompA2* by deleting an *Nde*I site which may have interfered with construction of the gene fusion. Because of the lack of convenient restriction sites, the PCR was used to modify the flanking ends of *phoA* in order to express this gene as part of a translational fusion with *gtfB.1* in the same vector as was used to express the CtxB fusion protein. The primer sequence chosen was based on the sequence of *phoA* (5). An *Nde*I site was incorporated into the sequence of *phoA* just downstream from DNA encoding the leader peptide. The *Bam*HI site was located at the 3' end of the transcriptional terminator of *phoA*. *E. coli* AW1043 was the host used to express plasmid-encoded protein (18). This strain contains a kanamycin-resistant transposon which has insertionally inactivated the chromosomal copy of *phoA*. The lack of expression of PhoA in this strain was confirmed by enzyme assay and immunoblotting analysis (data not shown). Both the PhoA and CtxB fusion proteins were expressed at high levels and were relatively easy to purify. Analysis of the fusion proteins by SDS-PAGE indicated that the monomeric form of each fusion protein was the size predicted after cleavage of the leader peptide sequence (Fig. 2). The PhoA fusion protein retained enzymatic activity, as was demonstrated by its ability to convert *p*-nitrophenyl phosphate to *p*-nitrophenol. The sequences of the GtfB.1 peptides on both the CtxB and PhoA fusion proteins were confirmed by N-terminal analysis (UAB Peptide Core Facility).

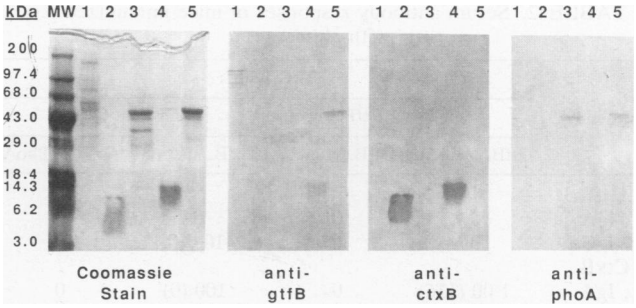

FIG. 2. Antigenicity of the chimeric proteins. Samples of the purified chimeras were compared with native GtfB, CtxB, and PhoA for antigenicity. Proteins were separated by denaturing SDS-PAGE on an 8 to 25% polyacrylamide gel. Replicate gels were separated simultaneously and electrophoretically transferred onto nitrocellulose membranes. One gel was stained with Coomassie blue and used to compare the proteins with standards of known molecular weight. The replicates were blotted with antiserum to GtfB, CtxB, or PhoA and stained to visualize immunoreactive protein. Lane (predicted molecular size): MW, molecular size markers; 1, GtfB (155 kDa); 2, CtxB (11.6 kDa); 3, PhoA (48 kDa); 4, GtfB.1::CtxB chimera (14.3 kDa); 5, GtfB.1::PhoA chimera (50.7 kDa).

Antigenicities of the fusion proteins. In order to determine whether the fusion proteins were still antigenic, each was analyzed by Western blot with antiserum specific for GtfB, CtxB, or PhoA. The results demonstrate that the fusion proteins retained the pattern of immunoreactivity predicted (Fig. 2). The CtxB chimera was immunoreactive with antibody to both CtxB and GtfB, and the PhoA chimera reacted with antibody to both PhoA and GtfB. This confirmed that the chimeras retained the desired antigenic properties and that they should be able to elicit an antibody response in vivo.

Comparison of oral immunogenicities of the chimeras. The abilities of the CtxB and PhoA chimeras to elicit antibody to the GtfB.1 peptide by oral feeding were compared. Because each molecule of CtxB is pentameric (16), the molar ratio of GtfB.1 to CtxB is 5:1. However, the molar ratio of GtfB.1 to PhoA is only 2:1, since PhoA normally exists as a homodimer (32). To ensure that the amounts of GtfB.1 peptide fed to the mice were identical, the doses of the chimeras were adjusted so that equimolar amounts of the peptide were delivered. This permitted the relative efficiency of each carrier to be compared. Two different doses of peptide were used: 6.9 nmol, which corresponded to 0.1 and 0.35 mg of the CtxB and PhoA chimeras, respectively, and 69 nmol, which corresponded to 1.0 and 3.5 mg of the CtxB and PhoA chimeras, respectively. The chimeras were administered to mice intragastrically by feeding needle on days 0, 10, and 20.

TABLE 2. Serum antibody responses of mice immunized p.o. with chimeras

Antibody[a]	Titer with[b]:			
	6.9 nmol of GtfB.1		69 nmol of GtfB.1	
	GtfB.1::CtxB	GtfB.1::PhoA	GtfB.1::CtxB	GtfB.1::PhoA
GtfB.1				
IgA	0	0	0	0
IgG	0	0	100 (0)	0
CtxB				
IgA	1 00 (116)	0	100 (0)	0
IgG	10,000 (0)	0	10,000 (0)	0
PhoA				
IgA	0	0	0	0
IgG	0	0	3,200 (0)	0

[a] Mice were immunized p.o. on days 0, 10, and 20. On day 27, the sera from each identically immunized group of mice (five mice per group) were collected and pooled. The amounts of IgA and IgG antibodies specific for GtfB.1, CtxB, and PhoA were determined by ELISA.
[b] Each pooled sample was assayed in triplicate. Results are reported as mean titers. Numbers in parentheses indicate standard deviations from the means.

TABLE 3. Intestinal and vaginal antibody responses of mice immunized p.o. with chimeras

Site and antibody[a]	Titer with[b]:			
	6.9 nmol of GtfB.1		69 nmol of GtfB.1	
	GtfB.1::CtxB	GtfB.1::PhoA	GtfB.1::CtxB	GtfB.1::PhoA
Intestinal				
GtfB.1				
IgA	0	0	0	0
IgG	0	0	0	0
CtxB				
IgA	8 (0)	0	32 (0)	0
IgG	0	0	0	0
PhoA				
IgA	0	0	0	0
IgG	0	0	0	0
Vaginal				
GtfB.1				
IgA	0	0	0	0
IgG	0	0	0	0
CtxB				
IgA	20 (0)	0	20 (0)	0
IgG	20 (0)	0	40 (0)	0
PhoA				
IgA	0	0	0	0
IgG	0	0	0	20 (0)

[a] Mice were immunized p.o. on days 0, 10, and 20. On day 27, the intestinal and vaginal washings from each identically immunized group of mice (five mice per group) were collected and pooled. The amounts of IgA and IgG antibodies specific for GtfB.1, CtxB, and PhoA were determined by ELISA.
[b] Each pooled sample was assayed in triplicate. Results are reported as mean titers. Numbers in parentheses indicate standard deviations from the means.

On day 27, the mice were bled and the levels of antibody in their sera were quantitated. As shown in Table 2, serum IgG antibody to the GtfB.1 peptide was elicited only when the peptide was fused to CtxB and fed at a dose of 1 mg. In addition, serum IgA and IgG antibodies to CtxB were elicited at both doses of the GtfB.1::CtxB chimera used. An IgG response to PhoA was elicited by 3.5-mg doses of the GtfB.1::PhoA chimera, but no antipeptide antibody was detected.

Mucosal antibody distribution. Antibody responses to the chimeras were determined at several mucosal sites in mice fed either GtfB.1::CtxB or GtfB.1::PhoA. Intestinal lavage fluid contained IgA antibody specific for CtxB, but no antibody to the peptide or to PhoA was detected (Table 3). IgA and IgG antibodies to CtxB were present in the vaginal washings of mice immunized with the CtxB chimera, but no antibody to the GtfB.1 peptide was detected (Table 3). However, unlike intestinal fluid, vaginal washings contained IgG antibody specific for PhoA in mice fed the highest dose of the PhoA chimera. The saliva and bronchial washings were void of any detectable levels of specific antibody to any of the antigens used for immunization, although immunoglobulin was present in the samples (data not shown).

Lack of oral tolerance to the chimeras. The lack of response to the GtfB peptide after the PhoA chimera was fed to the mice may have been due to the induction of oral tolerance. An experiment was conducted in order to determine whether antigen-specific oral tolerance was induced by any of the chimeras. The mice were fed 1.0 or 3.5 mg of the CtxB or PhoA chimera, respectively, on days 0, 10, and 20. On days 30 and 40, the mice were immunized i.p. with 10 μg of either chimera in 20% Maalox and bled on day 47. Serum IgG antibody titers were determined for GtfB.1, CtxB, and PhoA. Neither CtxB nor PhoA induced oral tolerance (Fig. 3). Furthermore, both carriers appeared equally efficient at inducing serum IgG antibody to themselves, whether administered orally or parenterally, at the doses used. Analysis of the response to the GtfB.1 peptide indicated that it did not induce oral tolerance either, regardless of which carrier was used (Fig. 4).

Antibody production in fragment cultures. The lack of detectable antibody to the GtfB.1 peptide in intestinal lavage fluid may have been due to dilution of the sample beyond the sensitivity of the assay. Lavage samples were already diluted at least 1:10 by the collection method used. Therefore, fragment cultures of the PP and MLN of mice orally immunized with the chimeras were prepared and assayed for antigen-specific antibody. By using this method, IgA and IgM antibodies to the GtfB.1 peptide were detected in PP and MLN after the GtfB.1::CtxB chimera was fed to the mice (Table 4). Although IgM antibody to GtfB.1 was being produced in MLN cultures derived from mice fed the GtfB.1::PhoA chimera, no antibody to PhoA itself was detected in either the PP or MLN cultures.

DISCUSSION

Several investigators have used CtxB in an effort to potentiate the immune response to antigens presented to the mucosal immune system; their success has been mixed (7, 21, 23, 26, 33). This situation is due in part to the variables in the model systems used. In an attempt to clarify the role of CtxB as a mucosal adjuvant, we constructed a set of chimeric proteins consisting of the same antigenic peptide sequence fused to either CtxB or an unrelated protein, PhoA. These fusion proteins were expressed in *E. coli*, which eliminated the possibility of contamination with CtxA. The relative oral immunogenicity of the same GtfB peptide on two different carriers was thus compared in a carefully controlled model system.

We chose PhoA as our alternate carrier protein for several reasons. First, *phoA* has been cloned (18) and sequenced (5), which made construction of the fusion protein relatively easy. Second, the crystal structure of the protein has been determined (32) and indicates that fusions to its N terminus

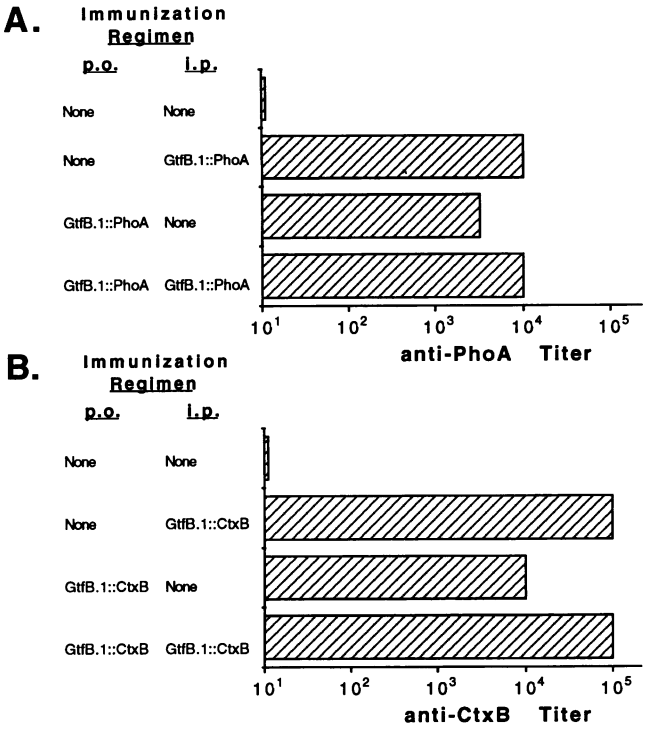

FIG. 3. Lack of oral tolerance to PhoA or CtxB. Mice (five per group) were fed (p.o.) either 1 or 3.5 mg of the CtxB or PhoA chimera, respectively, on days 0, 10, and 20. On days 30 and 40, they were injected i.p. with 10 μg of the chimera indicated. One week after the last dose i.p., the mice were bled and the sera were assayed for specific IgG antibody to PhoA (A) and CtxB (B).

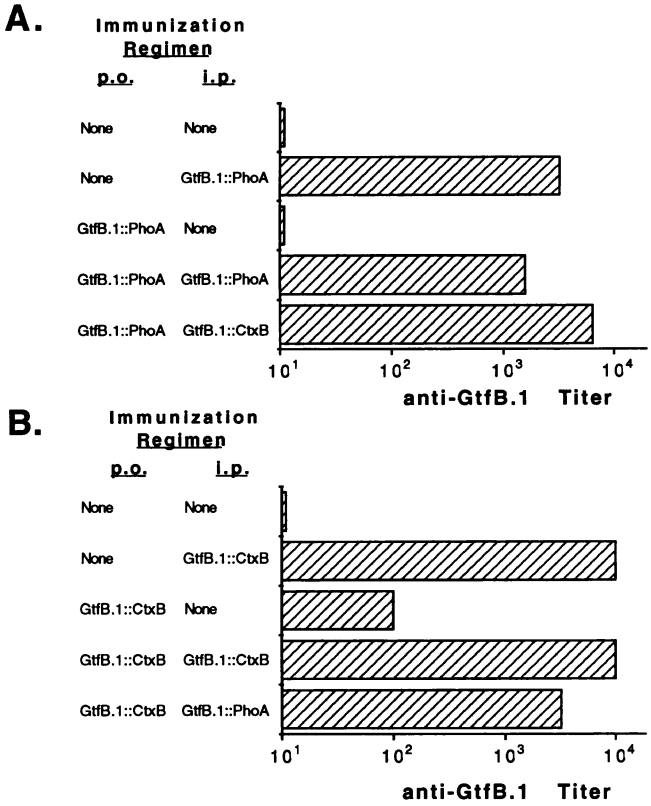

FIG. 4. Lack of oral tolerance to the GtfB.1 peptide on PhoA (A) or CtxB (B) as carrier. Mice (five per group) were fed (p.o.) either 1 or 3.5 mg of the GtfB.1::CtxB or GtfB.1::PhoA chimera, respectively, on days 0, 10, and 20. On days 30 and 40, they were injected i.p. with 10 μg of the chimera indicated. One week after the last dose i.p., the mice were bled and the sera were assayed for specific IgG antibody to the GtfB.1 peptide.

should have little effect on the structure of the protein. Third, the PhoA protein could be expressed in large quantities and was easy to isolate and purify. This was essential if enough protein was to be obtained for oral immunization. Fourth, *phoA* has little homology, either at the DNA or protein level, with its mammalian equivalent (19). This eliminated the possibility of self-tolerance, which was confirmed by the immunogenicity of native PhoA when administered i.p. (Fig. 3).

By using PCR, we were able to modify the N terminus of PhoA so that the GtfB.1 peptide sequence would be expressed on this protein as was done for CtxB. Furthermore, N-terminal modification enabled us to change the regulation of *phoA* expression so that expression was inducible. Secretion of the PhoA chimera into the periplasm of *E. coli* was achieved by using the *ompA* leader (15). Upon secretion, the leader was removed, and large quantities of the PhoA chimera could be isolated by osmotic shock and then purified by heat precipitation.

The peptide sequence we chose for making the fusions was derived from the *gtfB* gene of the cariogenic bacterium *S. mutans* (31), which encodes for an extracellular enzyme that converts sucrose into glucan polymer involved in the accumulation of the bacterium on the tooth surface. We have shown previously that this sequence, designated GtfB.1, is antigenic and that antiserum to this peptide inhibits enzyme activity in vitro (9). The lack of detectable antibody in the saliva, where the organism resides and where the antibody would be most needed, was disappointing. However, intranasal immunization (or boosting) of antigen coupled to CtxB may confer antibody in the saliva, because such delivery has been shown to stimulate antibody in the upper respiratory

TABLE 4. Antibody produced by fragment cultures of PP and MLN[a]

Antibody to[a]:	Mean titer[b] (μg/ml) in:			
	PP		MLN	
	GtfB.1::CtxB	GtfB.1::PhoA	GtfB.1::CtxB	GtfB.1::PhoA
GtfB.1				
IgG	0	0	0	0
IgA	0.20 (0.02)	0	3.37 (0.47)	0
IgM	41.7 (6.00)	0	37.0 (10.3)	24.2 (3.50)
CtxB				
IgG	66.8 (42.2)	0	771 (212)	0
IgA	2.60 (0.75)	0	34.9 (11.6)	0
IgM	68.4 (22.6)	0	30.1 (12.6)	0
PhoA				
IgG	0	0	0	0
IgA	0	0	0	0
IgM	0	0	0	0

[a] Mice were immunized p.o. on days 0 and 14 with either 1 or 3.5 mg of the GtfB.1::CtxB or GtfB.1::PhoA chimera, respectively. On day 21, tissue was harvested (five mice per group), pooled, and cultured as described in the text.

[b] Expressed as ratio of specific/total immunoglobulin. Each sample was assayed in triplicate. Numbers in parentheses indicate standard deviations from the means.

tract (33). This route is currently being explored as an alternative or adjunct to oral immunization.

The concept of a common mucosal immune system whereby induction of immunity at one site will result in the dissemination of antibody-secreting cells to distant mucosal sites has been generally acknowledged (25). However, our studies suggest that the common mucosal immune system is actually compartmentalized. There were no detectable levels of antibody to the peptide, CtxB, or PhoA in the saliva and bronchi of mice orally immunized with either chimera, but oral immunization did confer antibody in the sera, intestines, and vaginas of these mice. The presence of antibody to CtxB in the vagina suggests that oral immunization may be a practical method of conferring antibody at this site.

Oral tolerance, as classically defined, was not observed after the feeding of either the GtfB.1::CtxB or the GtfB.1::PhoA chimera, whether antibody was measured to the GtfB.1 peptide, CtxB, or PhoA (Fig. 3 and 4). Regardless of the carrier it was coupled to, the GtfB.1 peptide was able to induce serum antibody upon i.p. immunization. Feeding mice with the peptide on either carrier prior to i.p. challenge did not reduce the level of antibody produced compared with that produced by mice immunized i.p. only. An identical effect was observed for PhoA. The latter was surprising, since it is generally believed that most protein antigens induce tolerance upon oral feeding (4) and, with the exception of Ctx (12), do not generate a large serum antibody response after feeding, even in large amounts. This intriguing result suggests that despite the lack of antibody to PhoA in serum and secretions, there may have been low-level priming to this antigen, which is present in the gut continuously as a component of the normal flora.

Because of the presence of antipeptide antibody in the sera of mice fed the GtfB.1::CtxB chimera, we felt certain that the response had been generated in the gut-associated lymphoid tissue (GALT). The lack of detectable levels of antipeptide antibody in the intestinal secretions was attributed to the dilution of the sample by the collection method used and the sensitivity of the assay. To verify this, tissue fragments of PP and MLN from mice immunized with the chimeras were placed in culture, and the culture fluid was then assayed for specific antibody. As the serum antibody data in Table 2 reflect, the GtfB.1 peptide was most effective as an immunogen when coupled to CtxB (Table 4). Somewhat surprising was the response observed in the MLN. The PP is the major site of antibody induction in the GALT. Stimulated cells migrate from the PP, through the MLN, and into the circulation, where they are disseminated throughout the body. The large numbers of antibody-secreting cells located in the MLN suggest that a unique event that will either retain and/or activate these cells occurs in this tissue. These results also suggest that antipeptide antibody is likely to be present in the intestinal and vaginal secretions of the mice, where antibody to CtxB was found, but was simply below the limits of detection of the methods used. Use of a larger antigen with more determinants for elicitation and binding of antibody may have alleviated this problem.

The lack of response observed by some investigators using CtxB as an oral adjuvant may be due to several factors, some of which may be interrelated. First, CtxB has not always been coupled to the antigen (23), which may have prevented efficient presentation of the antigen to GALT. Second, in those cases in which the antigen was coupled to CtxB (7, 21), the conjugates were not examined for the effect of the coupling on the immunogenicity of CtxB. We have shown that even small peptides genetically fused to the N terminus of CtxB can reduce the immunogenicity of CtxB (8). Third, we demonstrate here that CtxB can stimulate immunity to antigens presented to GALT better than the unrelated bacterial protein, PhoA, can. This phenomenon is clearly due to some unique property of CtxB, since either CtxB or PhoA was equally efficient at inducing a systemic immune response to itself and to the GtfB.1 peptide fused to it. However, in order for oral administration of GtfB.1 with CtxB to be effective, a relatively large amount of protein had to be used compared with what has been reported for Ctx (21, 23). Whether this amount is required to stimulate immunity to other antigens presented orally is currently unknown. We have not evaluated the GtfB.1::CtxB chimera for its immunogenicity by other routes of administration, such as intranasal, and CtxB may be even more effective as an adjuvant when administered by such routes (33). Furthermore, we have not examined what effect, if any, the addition of free Ctx would have on the immunogenicity of the preparation.

It is possible that fusion of the GtfB.1 peptide to CtxB has reduced the immunogenicity of CtxB enough to increase the minimal effective dose required to elicit an immune response by the oral route. Whether administered orally or systemically, CtxB normally is a very potent immunogen. However, we have shown that even minor additions to the N terminus of CtxB can significantly reduce the immunogenicity of CtxB (8). Fusion and/or conjugation of even larger antigens to CtxB may seriously affect both the immunogenicity and the adjuvanticity of CtxB. Furthermore, this reduction in immunogenicity was correlated with a reduction in affinity for GM_1 ganglioside. We believe that part of the adjuvant activity of CtxB is due to its ability to bind to this ganglioside and to be selected out of the intestinal milleu for presentation to the GALT. In addition, Ctx and CtxB both have been shown to inhibit lymphocyte proliferation in vitro, which clearly suggests an ability to affect the regulation of lymphocyte function in a cyclic-AMP-independent manner (35). Whether this ability is due to a change in cytokine pattern or in the balance of T-cell-mediated suppression versus help is currently being explored.

In those cases in which Ctx and CtxB have been compared for their abilities to act as mucosal adjuvants in rodents, Ctx was clearly more effective and did not require the antigen to be coupled (21, 23). The reason for this difference is not known, but the difference may be due to the ability of Ctx to elevate levels of cyclic AMP. Although several papers have described the effect of Ctx on lymphocyte function (1, 3, 24), very little has been done with CtxB. We intend to compare the effects of Ctx and CtxB on local lymphocyte function in an effort to understand this difference in adjuvant strength. Although Ctx may be a more potent adjuvant than CtxB, the toxicity associated with the ingestion of Ctx would will probably preclude use of Ctx as an adjuvant in humans (unless the toxicity can be separated from the adjuvanticity of the protein).

Although perhaps not be as potent as Ctx, CtxB may still be a useful adjuvant for mucosally administered vaccines. By using genetic engineering, we have demonstrated that sufficient amounts of chimeric CtxB protein can be expressed and purified to do vaccination studies and that CtxB can indeed act as a mucosal adjuvant.

ACKNOWLEDGMENTS

We express our sincere gratitude to H. Bowdon for her expert technical assistance with the fragment cultures. We thank P. Freimuth for kindly providing us with the *phoA* host and vectors.

Special thanks to J. Chlebowski for his advice and the gift of the anti-PhoA monoclonal antibody. We also thank H. Kuramitsu for the anti-GtfB antisera and S. Michalek for the GtfB preparation.

This work was supported by NIH grants T32-AIO7051, DK28632, and UO1-AI28147.

REFERENCES

1. **Anastassiou, E. D., H. Yamada, D. T. Boumpas, G. C. Tsokos, G. Thyphronitis, J. Balow, and J. J. Mond.** 1992. Cell. Immunol. **140:**237–247.
2. **Boyer, H., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
3. **Bromander, A., J. Holmgren, and N. Lycke.** 1991. Cholera toxin stimulates IL-1 production and enhances antigen presentation by macrophages in vitro. J. Immunol. **146:**2908–2914.
4. **Challacombe, S., and T. Tomasi.** 1980. Systemic tolerance and secretory immunity after oral immunization. J. Exp. Med. **152:**1459–1472.
5. **Chang, C. N., W. J. Kuang, and E. Y. Chen.** 1986. Nucleotide sequence of the alkaline phosphatase gene of *Escherichia coli*. Gene **44:**121–125.
6. **Chlebowski, J. (Virginia Commonwealth University).** 1989. Personal communication.
7. **Czerkinsky, C., M. W. Russell, N. Lycke, M. Lindblad, and J. Holmgren.** 1989. Oral administration of a streptococcal antigen coupled to cholera toxin B subunit evokes strong antibody responses in salivary glands and extramucosal tissues. Infect. Immun. **57:**1072–1077.
8. **Dertzbaugh, M. T., and C. O. Elson.** Reduction in oral immunogenicity of cholera toxin B subunit by N-terminal peptide addition. Submitted for publication.
9. **Dertzbaugh, M. T., and F. L. Macrina.** 1990. Inhibition of *Streptococcus mutans* glucosyltransferase activity by antiserum to a subsequence peptide. Infect. Immun. **58:**1509–1513.
10. **Dertzbaugh, M. T., D. L. Peterson, and F. L. Macrina.** 1990. Cholera toxin B-subunit gene fusion: structural and functional analysis of the chimeric protein. Infect. Immun. **58:**70–79.
11. **Elson, C. O., and W. Ealding.** 1984. Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. J. Immunol. **132:**2736–2741.
12. **Elson, C. O., and W. Ealding.** 1984. Cholera toxin feeding did not induce oral tolerance in mice and abrogated oral tolerance to an unrelated protein antigen. J. Immunol. **133:**2892–2897.
13. **Elson, C. O., W. Ealding, and J. Lefkowitz.** 1984. A lavage technique allowing repeated measurement of IgA antibody in mouse intestinal secretions. J. Immunol. Methods **67:**101–108.
14. **Freimuth, P. (Rockefeller University).** 1989. Personal communication.
15. **Ghrayeb, J., H. Kimura, M. Takahara, H. Hsiung, Y. Masui, and M. Inouye.** 1984. Secretion cloning vectors in *Escherichia coli*. EMBO J. **3:**2437–2442.
16. **Gill, D. M.** 1976. The arrangement of subunits in cholera toxin. Biochemistry **15:**1242–1248.
17. **Guerry, P., D. J. LeBlanc, and S. Falkow.** 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. **116:**1064–1066.
18. **Inouye, H., S. Michaelis, A. Wright, and J. Beckwith.** 1981. Cloning and restriction mapping of the alkaline phosphatase structural gene (*phoA*) of *Escherichia coli* and generation of deletion mutants in vitro. J. Bacteriol. **146:**668–675.
19. **Kam, W., E. Clauser, Y. S. Kim, Y. W. Kan, and W. J. Rutter.** 1985. Cloning, sequencing, and chromosomal localization of human term placental alkaline phosphatase cDNA. Proc. Natl. Acad. Sci. USA **82:**8715–8719.
20. **Laemmli, U. K.** 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
21. **Liang, X., M. Lamm, and J. Nedrud.** 1988. Oral administration of cholera toxin-Sendai virus conjugates potentiates gut and respiratory immunity against Sendai virus. J. Immunol. **141:** 1495–1501.
22. **Logan, A. C., K. N. Chow, A. George, P. D. Weinstein, and J. J. Cebra.** 1991. Use of Peyer's patch and lymph node fragment cultures to compare local immune responses to *Morganella morganii*. Infect. Immun. **59:**1024–1031.
23. **Lycke, N., and J. Holmgren.** 1986. Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. Immunology **59:**301–308.
24. **Lycke, N., E. Severinson, and W. Strober.** 1990. Cholera toxin acts synergistically with IL-4 to promote IgG1 switch differentiation. J. Immunol. **145:**3316–3324.
25. **McGhee, J. R., and S. M. Michalek.** 1981. Immunobiology of dental caries: microbial aspects and local immunity. Annu. Rev. Microbiol. **35:**595–638.
26. **McKenzie, S. J., and J. F. Halsey.** 1984. Cholera toxin B subunit as a carrier protein to stimulate a mucosal immune response. J. Immunol. **133:**1818–1824.
27. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 431–435. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Morrison, D. A.** 1977. Transformation in *Escherichia coli*: cryogenic preservation of competent cells. J. Bacteriol. **132:** 349–351.
29. **Nedrud, J. G., X. Liang, N. Hague, and M. E. Lamm.** 1987. Combined oral/nasal immunization protects mice from Sendai virus infections. J. Immunol. **139:**3483–3492.
30. **Pierce, N. F., and J. L. Gowans.** 1975. Cellular kinetics of the intestinal immune response to cholera toxoid in rats. J. Exp. Med. **142:**1550–1563.
31. **Shiroza, T., S. Ueda, and H. K. Kuramitsu.** 1987. Sequence analysis of the *gtfB* gene from *Streptococcus mutans*. J. Bacteriol. **169:**4263–4270.
32. **Sowadski, J. M., M. D. Handschumacher, H. M. K. Murthy, B. A. Foster, and H. W. Wyckoff.** 1985. Refined structure of alkaline phosphatase from *Escherichia coli* at 2.8 A resolution. J. Mol. Biol. **186:**417–433.
33. **Tamura, S., Y. Samegai, H. Kurata, T. Nagamine, C. Aizawa, and T. Kurata.** 1988. Protection against influenza virus infection by vaccine inoculated intranasally with cholera toxin B subunit. Vaccine **6:**409–413.
34. **Welch, R. A., K. R. Jones, and F. L. Macrina.** 1979. Transferable lincosamide-macrolide resistance in *Bacteroides*. Plasmid **2:**261–268.
35. **Woogen, S. D., W. Ealding, and C. O. Elson.** 1987. Inhibition of murine lymphocyte proliferation by the B subunit of cholera toxin. J. Immunol. **139:**3764–3770.