A Recombinant Live Attenuated Strain of *Vibrio cholerae* Induces Immunity against Tetanus Toxin and *Bordetella pertussis* Tracheal Colonization Factor

INÊS CHEN,[1,2] THERESA M. FINN,[3] LIU YANQING,[4] QI GUOMING,[4]
RINO RAPPUOLI,[1]* AND MARIAGRAZIA PIZZA[1]

*IRIS, Chiron Vaccines Immunobiological Research Institute in Siena, 53100 Siena, Italy[1]; Departamento de
Microbiologia, ICB II, Universidade de São Paulo, São Paulo, SP 05508-900, Brazil[2]; Laboratory of
Pertussis, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda,
Maryland 20892-0029[3]; and Chinese Academy of Preventive Medicine, Beijing, China[4]*

An attenuated strain of *Vibrio cholerae* was used as a carrier for the expression of heterologous antigens such as fragment C from tetanus toxin (TetC) and tracheal colonization factor from *Bordetella pertussis* (Tcf). In vitro, high levels of protein were obtained when the *Escherichia coli nirB* promoter was used and the bacteria were grown with low aeration. Intranasal immunization of mice with IEM101 expressing TetC elicited serum vibriocidal activity and induced antibodies against tetanus toxin which were protective against lethal challenge with 10 times the 50% lethal dose of tetanus toxin. Bacterial viability was essential for the induction of anti-TetC antibodies. Intranasal administration of IEM101 expressing Tcf induced a significant reduction in bacterial colonization of the tracheas of mice challenged with wild-type *B. pertussis*. These data are in agreement with the putative role of Tcf in *Bordetella* tracheal colonization. In conclusion, we have demonstrated that *V. cholerae* may be used as a live vector to deliver heterologous antigens in vivo and that protection to both systemic and local challenge may be achieved.

Vibrio cholerae is the causative agent of cholera, a human diarrheal disease with high rates of morbidity and mortality in developing countries. Infection occurs after ingestion of contaminated water or food; the bacteria reach the small intestine, where they penetrate the mucous layer, adhere to epithelial cells, multiply, and produce cholera toxin. This powerful toxin induces secretion of chloride ions by enterocytes, leading to a severe watery diarrhea (3).

Naturally acquired infection with *V. cholerae* efficiently stimulates immunity, leading to long-term protection against the disease (24). In the last years, live attenuated oral vaccines against *V. cholerae* have been developed (9, 25, 30, 35). A naturally attenuated strain of *V. cholerae* O1 El Tor, Ogawa, isolated in China (IEM101) has been also described (26). IEM101 has been tested for safety and immunogenicity in a human clinical study, which showed that the strain is safe and able to colonize the intestinal mucosa and to induce a strong immune response, eliciting high levels of vibriocidal and anti-lipopolysaccharide antibodies in the sera of human volunteers. Due to these characteristics and to the noninvasive nature of *V. cholerae* infection, attenuated strains of *V. cholerae* may be good candidates for the delivery of foreign antigens to the host.

Previous work on heterologous expression in *V. cholerae* has used antigens from other enteropathogens, such as *Shigella sonnei* lipopolysaccharide, *Escherichia coli* Shiga toxin B subunit, and enterohemorrhagic *E. coli* EaeA (1, 4, 6, 13, 36). In this study, we have investigated whether the attenuated strain IEM101 is able to express antigens from nonenteric pathogens and whether these recombinant strains induce protective immunity to systemic and mucosal challenge.

The choice of an animal model to evaluate immune responses induced by recombinant *V. cholerae* strains is a critical step, since natural infection with *V. cholerae* does not occur in animals. Various animal models have been used in cholera research (33). Infant mice or rabbits are susceptible to infection for a short time after birth; however, given the immature immune system of neonatal animals, they do not constitute a good model for immunogenicity studies. Colonization by *V. cholerae* has been accomplished in adult rabbits, with tincture of opium used to induce paralysis of intestinal motility, but this model presents some limitations in terms of animal handling. It has been recently described that germfree mice are readily colonized by *V. cholerae* after oral inoculation (5); however, these animals are usually more expensive than nongermfree ones.

The intranasal route has been shown to be highly efficient for the induction of immune responses, at the systemic and mucosal levels, with a variety of antigens delivered, either with mucosal adjuvants (10, 11, 37) or microcapsules (21). This route has also been used to deliver live *Salmonella typhimurium* (19), *Bordetella pertussis* (32), *Mycobacterium bovis* BCG (22), and even *Salmonella typhi*, which also lacks a practical small-animal model (2, 16).

In this study we have used intranasal immunization as an alternative mucosal route. Fragment C (TetC) from tetanus toxin (TT) and tracheal colonization factor (Tcf) from *B. pertussis* were used as model antigens. TetC is the nontoxic 50-kDa C-terminal portion of TT (18); it is immunogenic and able to protect against challenge with the toxin when either administered as purified immunogen (12) or delivered by attenuated *Salmonella* (8). Tcf is a virulence-associated factor secreted by *B. pertussis*, and *tcfA* mutants are impaired in their ability to colonize the mouse trachea after aerosol infection (15). Tcf is produced as a cell-associated precursor form, with an apparent molecular mass of 90 kDa, which is processed to release the 60-kDa form.

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* Corresponding author. Mailing address: IRIS, Chiron Vaccines Immunobiological Research Institute in Siena, Via Fiorentina, 1, 53100 Siena, Italy. Phone: 39-577-243414. Fax: 39-577-243564. E-mail: Rappuoli@IRIS02.BIOCINE.IT.

MATERIALS AND METHODS

Strains, media, and growth conditions. *E. coli* DH5α was used for cloning purposes. *V. cholerae* IEM101 was grown in Luria broth or Luria broth with ampicillin (100 μg/ml) when required. *B. pertussis* BP18323 Sm^r was grown in Stainer-Scholte (SS) modified medium (34) or on Bordet-Gengou (BG) agar (Difco) with 20% defibrinated sheep blood and streptomycin (40 μg/ml). Transformation of IEM101 was performed by electroporation as previously described (17). Growth of bacterial cultures under low-aeration conditions was achieved by inoculation of 2 ml from an overnight culture into a completely filled, tight-capped 50-ml Falcon tube and subsequent incubation at 37°C for 3.5 to 4 h. To recover IEM101 from organ homogenates, TCBS agar (Difco) was used.

Plasmids and constructions. Plasmid pTETnir15 (obtained from G. Dougan, Imperial College, London, United Kingdom) contains the coding sequence for TetC under the control of the *E. coli nirB* modified promoter, which is induced under anaerobic conditions (29).

tcfA was amplified from the *B. pertussis* BP18323 Sm^r chromosome by using *Taq* DNA polymerase (Boehringer Mannheim). The forward primer was oligonucleotide Tcf1 (5′-ACTAGTGATCATATGCACAATTTACGGAAATA-3′), which contains the initial codon of *tcfA*, comprised within the *Nde*I site, and an upstream *Bcl*I site; the reverse primer was oligonucleotide Tcf2 (5′-GTCTAGA ATTCTACCAGGCGTAGCGATACC-3′), containing the stop codon of the *tcfA* gene and an *Eco*RI site downstream. The amplification product was digested with *Bcl*I and *Eco*RI and cloned into pBlueScript/KS+ (Stratagene) between *Eco*RI and *Bam*HI sites, giving origin to pBS-tcf. The cloned fragment was completely sequenced, and one mutation, leading to a conservative amino acid substitution (Met to Thr) in position 494, probably due to *Taq* DNA polymerase inaccuracy, was found.

tcfA was placed under the control of the cholera toxin gene promoter (p*ctx*) as follows: a 190-bp sequence located immediately upstream to the *ctx* structural region was amplified from plasmid pJM17, which contains the virulence cassette region from *V. cholerae* classical strain 569B (31). Oligonucleotides Pct1 (5′-TA GATCTAGATACCTTTGCAGCGCAAGG-3′) and Pct2 (5′-TATCTTTACCA TATGATGCTCCC-3′) were used as forward and reverse primers, respectively; Pct1 contains an *Xba*I site at its 5′ extremity, whereas Pct2 corresponds to the reverse sequence of the *ctx* gene, with the initiation codon (reverse) within an *Nde*I site. The amplified fragment was digested with *Xba*I and *Nde*I and cloned into pBS-tcf digested with the same enzymes, generating pCT-tcf.

Subcloning *tcfA* downstream of the *nirB* promoter from pTETnir15 was achieved in two steps. First, pTETnir15 was digested with *Bgl*II and *Bam*HI, liberating a fragment containing the coding region for TetC and ca. 30 bp of the 5′ untranslated region, including the putative ribosomal binding site sequence. In order to reconstitute this region, which could be important for RNA stability and/or efficient translation initiation, the fragment containing the vector sequence was ligated to oligonucleotides PnirUTR1 (5′-GATCTTAATCATCCA CAGGAGACTTTCATATGATATCTAGATGCATC-3′) and PnirUTR2 (5′-G ATCGATGCATCTAGATATCATATGAAAGTCTCCTGTGGATGATTAA-3′), corresponding to the 5′ untranslated region, with an *Nde*I site comprising the initiation codon, and an *Xba*I site immediately downstream. This plasmid was called pNIR100; it was digested with *Xba*I, filled in with Klenow fragment from *E. coli* DNA polymerase I, digested with *Nde*I, and ligated to the *Nde*I-*Eco*RV fragment from pBS-tcf, which contains the *tcfA* gene, generating pNIR-tcf.

Analysis of protein expression and localization. Expression of TetC was analyzed by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS–12.5% PAGE) and immunoblotting of whole-cell lysates, using mouse anti-TT antiserum at a dilution of 1:2,000 (obtained from S. Peppoloni, IRIS, Siena, Italy).

Tcf expression was probed by immunoblotting, using mouse antiserum raised against the N-terminal portion of Tcf fused to MalE (15) at a dilution of 1:8,000. Culture supernatant (1 ml) from *B. pertussis* or *V. cholerae* was trichloroacetic acid precipitated and loaded on an SDS-polyacrylamide gel. *B. pertussis* whole-cell lysate was prepared by pelleting bacteria from a liquid culture with an optical density at 590 nm (OD$_{590}$) of 1.0 and resuspension in 1× sample boiling buffer in order to have approximately 5×10^9 cells/ml. *B. pertussis* outer membrane-associated proteins (OMAP) were prepared by resuspending cells in 50 mM Tris-HCl (pH 8.0)–150 mM NaCl to a final concentration of 20 OD units/ml; the suspension was incubated at 60°C for 1 h with gentle shaking; intact cells were pelleted by centrifugation, and the supernatant, containing the OMAP, was used for SDS-PAGE. Whole-cell lysates of IEM101 were prepared by pelleting cells from liquid cultures and resuspending them in sample boiling buffer to a final concentration of approximately 10^{10} cells/ml; the *V. cholerae* outer membrane fraction was prepared from 10^9 cells, as previously described (14).

FACScan analysis was used to probe Tcf exposure on the bacterial cell surface. Approximately 10^6 cells were pelleted and kept on ice through the procedure. Cells were washed with phosphate-buffered saline (PBS)–2% bovine serum albumin (BSA) and incubated for 1 h with serial dilutions of anti-MalE-Tcf mouse antisera in 200 μl of PBS–2% BSA; mouse preimmune serum was used as a negative control. Bacterial cells were washed twice with PBS–2% BSA and incubated for 30 min with the appropriate dilution of R-phycoerythrin-labeled F(ab′)$_2$ goat anti-mouse immunoglobulin G (IgG) antiserum. Cells were subsequently washed twice with PBS–2% BSA and resuspended in PBS, and 10^4 bacterial cells were analyzed for cell-bound fluorescence with a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.), using the Lysis II software program from Becton Dickinson. The threshold of positivity was set for each experiment by flow cytometric analysis of IEM101 previously incubated with antisera to MalE-Tcf and with the R-phycoerythrin-labeled secondary antibody.

Immunizations. Female BALB/c mice (6 to 8 weeks old; Charles River, Calco, Italy) each received 30 μl of a bacterial suspension in PBS by the intranasal route, either without anesthesia or anesthetized with 0.2 ml of a mixture of 15% xylazine hydrochloride (Rompun) and 10% ketamine hydrochloride (Ketavet), on days 0, 28, 42, and 56. Animals were bled on days 27, 35, 49, and 63 and challenged at day 70. Nasal lavages were performed at day 70 by repeated flushing and aspiration with 1 ml of PBS containing 0.1% BSA (Sigma).

Titration of antigen-specific serum antibodies. Vibriocidal activity was assayed by incubating 10^7 CFU of IEM101 with 20% rabbit serum as the complement source and serial dilutions of immunized animal sera in 100 μl of PBS in 96-well tissue culture plates (Costar), for 1 h at 37°C; 100 μl of brain heart infusion (BHI) (Difco) was then added to each well, and plates were further incubated for 1 h at 37°C; absorbance at 570 nm was then measured. Titers were calculated as the dilution of the serum that gave 50% growth inhibition compared to preimmune serum diluted 1:25.

Titers of anti-TetC antibodies were determined by enzyme-linked immunosorbent assay (ELISA). Plates were coated overnight at 4°C with 200 ng of tetanus toxoid (formaldehyde-inactivated TT) in 100 μl of PBS/well. After three washes with PBS–0.05% Tween 20 (PBS-T), plates were blocked with 1% BSA in PBS-T (200 μl/well) for 2 h at 37°C, washed three times with PBS-T, and incubated with serial dilutions of sera from immunized animals in PBS-T–0.5% BSA for 1.5 h at 37°C; plates were then incubated with PBS-T–0.5% BSA containing alkaline phosphatase-conjugated anti-mouse IgG antibody (Sigma) for 1.5 h at 37°C. Bound antibodies were revealed by using *p*-nitrophenylphosphate as a substrate (Sigma). Titers were calculated as the dilution that gave 2.5 times the absorbance (OD$_{405}$) of preimmune serum diluted 1:100.

Mucosal IgA titers were measured by using biotin-conjugated goat anti-mouse IgA (α-chain specific; Sigma) followed by streptavidin-horseradish peroxidase conjugate (Dako). Bound antibodies were revealed by using *o*-phenylenediamine as a substrate. Titers were determined as the dilution that gave 2.5 times the absorbance (OD$_{490}$) of nasal washes of nonimmunized animals.

TT challenge. Mice were challenged subcutaneously with 10 times the 50% lethal dose ($10 \times$ LD$_{50}$) of TT. Paralysis and death were recorded for 7 days after the challenge.

***Bordetella* intranasal challenge.** *B. pertussis* 18323 Sm^r was grown for 2 days on a BG plate and then inoculated into 100 ml of SS modified medium; the culture was grown until it reached an OD$_{590}$ of 0.5. Bacteria were then diluted in PBS to a concentration of 3.3×10^8 CFU/ml; the concentration was confirmed by serial dilution and plating. Animals received 30 μl of the bacterial suspension (corresponding to approximately 10^6 CFU) intranasally, under light anesthesia. Colonization of the trachea and lungs was monitored by counting CFU recovered from organ homogenates.

RESULTS

Expression of TetC in IEM101. The plasmid pTETnir15, which contains the gene coding for TetC under the control of the modified *E. coli nirB* promoter, was electroporated into *V. cholerae* IEM101. Total cell extracts were prepared from both IEM101 and IEM101(pTETnir15) strains and analyzed by Western blotting. As shown in Fig. 1, transformants produced TetC, and expression was induced when bacteria were grown with low aeration, indicating that the *nirB* promoter was functional in IEM101.

Expression and localization of *tcfA* in IEM101. *tcfA* was subcloned under the control of the *ctx* promoter (pCT-tcf) and *nirB* promoter (pNIR-tcf); the recombinant plasmids were electroporated into IEM101. Total cell extracts were prepared from each of the recombinant strains, and the levels of Tcf were visualized by immunoblotting. The results are shown in Fig. 2.

Tcf production was observed when either the *ctx* or *nirB* promoters were used (Fig. 2, lanes c and d, respectively). The highest level of expression was achieved when the p*nirB* construct was induced by growth with low aeration (Fig. 2, lane e).

In order to analyze Tcf localization in the bacteria, we performed immunoblotting experiments using different cellular fractions, and the results are reported in Fig. 3. In *B. pertussis*, Tcf could be found in the culture supernatant (Fig. 3, lane b), confirming previously reported data (15), and in the OMAP preparation (Fig. 3, lane a), which indicates that it is loosely
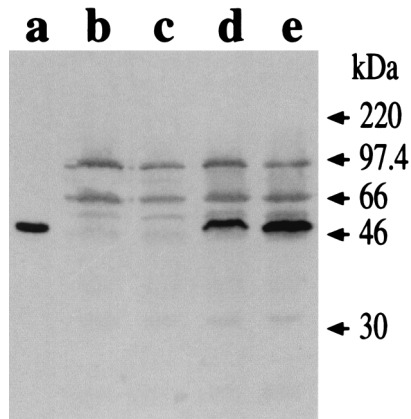
FIG. 1. TetC expression by IEM101. Whole-cell lysates (corresponding to approximately 2.5×10^8 CFU of bacteria) were fractionated by SDS-PAGE and probed with anti-TT mouse polyclonal antiserum. Purified TetC (100 ng) (lane a) was used as a standard. IEM101 was grown under aerobic conditions (lane b) or low-aeration conditions (lane c); the same conditions were used for growing IEM101(pTETnir15) (lane d, aerobic; lane e, low aeration).

associated with the outer membrane. In *V. cholerae*, Tcf localized in the outer membrane fraction (Fig. 3, lane d), although small amounts could be detected in the culture supernatant (Fig. 3, lane e). Tcf appears to be processed at a different site in *V. cholerae*, since the molecular mass of the protein appears to be less than that of the 60-kDa form detected in *Bordetella* extracts and supernatant (Fig. 3, lanes a and b, respectively).

Flow cytometry analysis was performed to verify whether Tcf was exposed on the *V. cholerae* surface. The percentage of cells emitting fluorescence was 31% in the case of *B. pertussis* and 14% in the case of IEM101(pNIR-tcf), suggesting that the protein is less exposed in *V. cholerae* than in *B. pertussis* (data not shown).

Intranasal administration of *V. cholerae*. The intranasal route was chosen to deliver *V. cholerae* to mice since it has been reported to be more efficient than the oral route in inducing an immune response against foreign antigens expressed by live recombinant bacterial strains (16, 19).
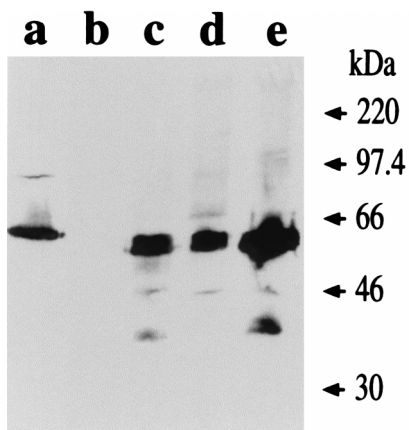
Bacterial administration conditions were defined. Animals

FIG. 2. Tcf expression by IEM101 under control of different promoters. Whole-cell lysates (corresponding to approximately 5×10^8 CFU of IEM101 or 5×10^7 CFU of BP18323) were fractionated by SDS-PAGE and probed with anti-Tcf antiserum. Samples shown are BP18323 (lane a), IEM101 (lane b), IEM101(pCT-tcf) (lane c), IEM101(pNIR-tcf) grown under aerobic conditions (lane d), and IEM101(pNIR-tcf) grown with low aeration (lane e).
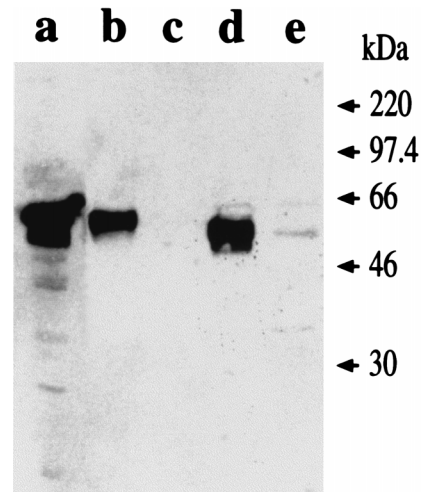

FIG. 3. Tcf localization in IEM101. OMAP preparations from *B. pertussis* 18323 (from approximately 2×10^8 CFU), outer membrane protein fractions from IEM101 (from approximately 10^9 CFU), and trichloroacetic acid-precipitated culture supernatant (corresponding to 1 ml) were loaded on SDS-polyacrylamide gels and probed with anti-Tcf antiserum. Samples shown are *B. pertussis* 18323 OMAP (lane a), *B. pertussis* 18323 culture supernatant (lane b), IEM101 outer membrane fraction (lane c), IEM101(pNIR-tcf) outer membrane fraction (lane d), and IEM101(pNIR-tcf) culture supernatant (lane e).

under anesthesia could receive intranasally up to 5×10^7 CFU without mortality. A higher bacterial dose (5×10^8 CFU) could be delivered either by inoculation of nonanesthetized animals or by using heat-inactivated bacteria. Both treatments resulted in similar serum vibriocidal activity in both groups, as presented in Fig. 4. The titers in mice inoculated with live bacteria were slightly higher than those in mice inoculated with
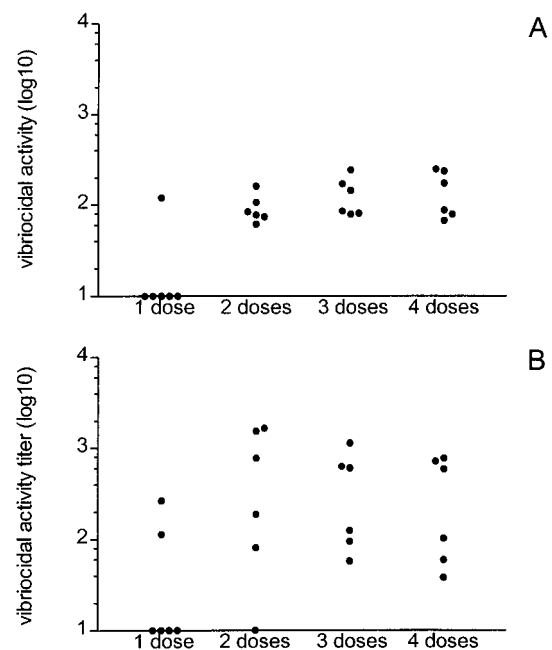

FIG. 4. Serum vibriocidal activities. Serum vibriocidal activities in BALB/c mice after receiving one to four doses of 5×10^8 bacteria, either heat inactivated (A) or live (B), with six animals per group, are presented. Results are shown as individual values of $\log_{10}$ vibriocidal activity titers.
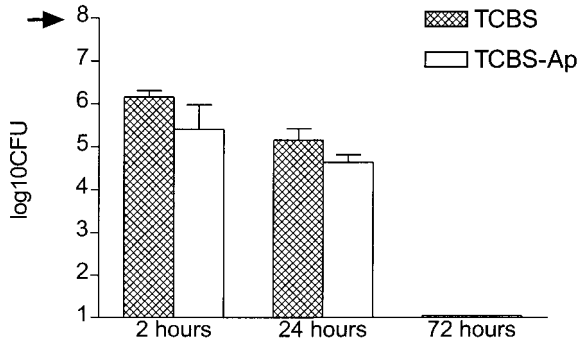
FIG. 5. IEM101(pTETnir15) persistence in vivo. BALB/c mice received intranasally 10^8 CFU of IEM101(pTETnir15) (dose indicated by arrow on *y* axis); the presence of bacteria in the mouse trachea was assessed after 2, 24, and 72 h, with three animals sacrificed per time point. Bacterial counts were performed on TCBS agar plates, with (TCBS-Ap) or without (TCBS) ampicillin. Results are shown as means + standard deviations (error bars) of log_{10} CFU/organ.

FIG. 7. Anti-TT mucosal IgA response. Anti-TT IgA antibody responses in nasal washes of five BALB/c mice after receiving four doses of 5×10^8 CFU of either IEM101 or IEM101(pTETnir15) are presented. Results are shown as mean titers, and the error bar indicates the standard deviation from the mean titer.

heat-inactivated bacteria, but the difference was not statistically significant.

After one intranasal administration of 5×10^8 CFU IEM101 (pTETnir15) to nonanesthetized animals, bacterial persistence in trachea, lung, small intestine, and stool was analyzed. The results, reported in Fig. 5, show that viable bacteria could be recovered from mouse tracheas 24 h after inoculation, and furthermore, most of the recovered bacteria could be cultured on ampicillin plates, showing that bacteria had not lost the plasmid. However, only some of the animals presented bacteria in their lungs (not shown). Bacteria could also be recovered from small intestine and stool, possibly due to the animals' swallowing inoculum during administration. No bacteria were detected in any organ after 72 h.

Induction of anti-TT antibody response and in vivo protection after immunization with IEM101-delivered TetC. Nonanesthetized BALB/c mice received four doses of ca. 5×10^8 CFU of either IEM101 or IEM101(pTETnir15), grown without aeration, by the intranasal route. A third group of mice received the same dose of heat-inactivated IEM101 (pTETnir15). Levels of humoral anti-TT IgG were analyzed by ELISA. When killed bacteria were used for immunization, no anti-TT IgG response was detected in the sera of the immunized mice. After three immunizations, mice immunized with live bacteria mounted an anti-TT humoral response, which was boosted following the fourth immunization (Fig. 6). An an-
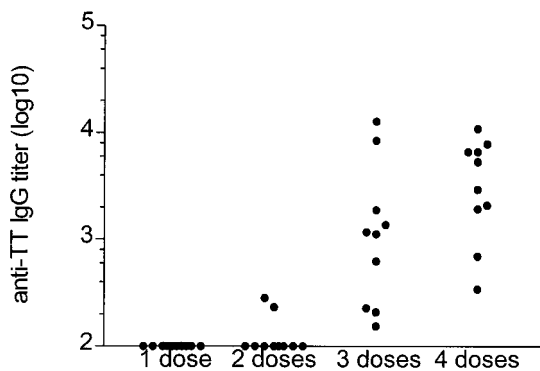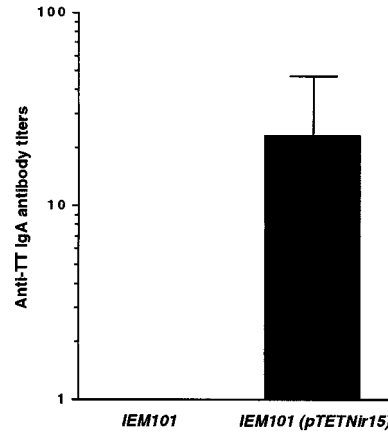
ti-TT IgA response could be also detected in nasal washes, indicating that stimulation of local responses occurred (Fig. 7).

To verify whether the induced anti-TT antibodies were able to protect immunized mice against a lethal challenge with tetanus toxin, mice were challenged with $10 \times LD_{50}$ of tetanus toxin on day 70 and observed for mortality for 7 days after the challenge. The results, reported in Fig. 8, show that all mice immunized with IEM101(pTETnir15) survived the challenge, whereas control animals, immunized with IEM101, died within 36 h, demonstrating that the immune response raised by *Vibrio*-delivered TetC was able to neutralize the toxin in vivo and confer protection.

In vivo protection following immunization with IEM101-delivered Tcf. BALB/c animals received four doses of ca. 5×10^8 CFU of either IEM101 or IEM101(pNIR-tcf) by the intranasal route. We were unable to detect specific anti-Tcf responses in the sera and mucosal washes of immunized mice, either by ELISA or Western blotting. Purified recombinant MalE-Tcf and partially purified Tcf from *B. pertussis* were used as antigens for the assays, but sera of mice immunized with
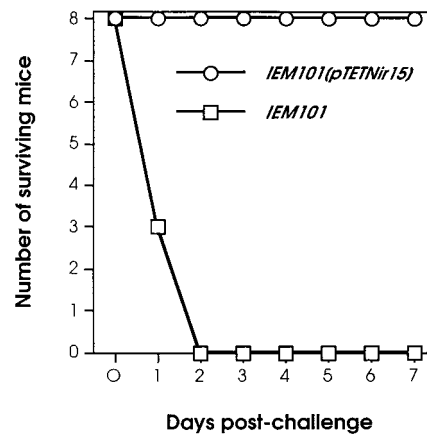

FIG. 6. Anti-TT serum antibody responses. Anti-TT serum antibody responses in 10 BALB/c mice after receiving one to four doses of 5×10^8 CFU of IEM101(pTETnir15) are presented. Results are shown as individual values of log_{10} anti-TT IgG.

FIG. 8. Mouse survival after challenge with TT. Eight mice were immunized with four doses of 5×10^8 CFU of either IEM101 or IEM101(pTETnir15). At day 70, mice were challenged with $10 \times LD_{50}$ of tetanus toxin and observed for mortality for 7 days after challenge.
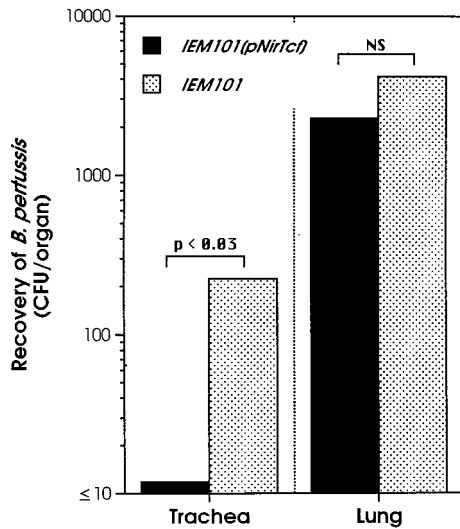
FIG. 9. *B. pertussis* colonization in trachea and lung following intranasal infection. Six mice were immunized with four doses of 5×10^8 CFU of either IEM101 or IEM101(pNIR-tcf). At day 70, mice were challenged intranasally with 10^6 CFU of BP18323. Fourteen days after challenge, the mice were sacrificed and colonization in lungs and tracheas was determined. Results are expressed as geometrical means of bacterial counts. Values are significantly different, with a *P* of <0.03. NS, values not significantly different.

IEM101 also recognized such preparations. These background signals could be due to a response to endogenous MalE from IEM101 or cross-reaction with proteins copurified with Tcf.

Although it was impossible to detect a specific anti-Tcf antibody response, we asked whether mice immunized with IEM101-delivered Tcf were protected from tracheal colonization by *B. pertussis*, since it has been described that a *tcf* mutant *B. pertussis* strain showed decreased tracheal colonization, implying a possible role for this protein in tracheal colonization (15). On day 70, immunized animals were challenged via the intranasal route with BP18323 Sm^r. Mice were sacrificed 14 days after the challenge, and the bacterial colonization of their tracheas and lungs was evaluated. The results, shown in Fig. 9, indicate that mice immunized with IEM101(pNIR-tcf) had significantly less bacteria in their tracheas than did the control animals immunized with IEM101. No significant difference between the numbers of bacteria isolated from the lungs of the two groups of mice was observed.

DISCUSSION

The construction of attenuated *V. cholerae* strains as live oral vaccines for cholera raises the possibility of using such strains to deliver heterologous antigens and to develop multivalent vaccines. We have thus expressed two antigens from nonenteropathogens in a naturally attenuated strain of *V. cholerae*, IEM101, and investigated its potential as a delivery system.

TetC was expressed in IEM101 by using pTETnir15 (29). Bacteria administered intranasally to mice induced a serum vibriocidal response and anti-TetC antibodies and stimulated local responses to the foreign protein. An anti-TT response able to confer protection against lethal challenge with TT was achieved.

Interestingly, immunization with heat-inactivated bacteria did not raise antibodies against TetC, although there was induction of vibriocidal activity, at levels comparable to those induced by live bacteria. Bacterial viability was shown to be necessary for *V. cholerae* interaction with M cells in rabbit

intestine (28). It is probable that bacterial viability is also needed for interaction with M cells from respiratory mucosa; therefore, live bacteria would interact with M cells more efficiently than inactivated bacteria and consequently induce a stronger immune response. Persistence of IEM101 in the respiratory tract after intranasal inoculation (for at least 24 h) could also provide a more effective stimulation of the immune system. Plasmid retention was considerably high; thus, in vivo expression of the heterologous antigen could potentially occur. However, no significant differences in vibriocidal antibody titers in sera from animals receiving live and heat-inactivated *Vibrio* bacteria were observed; it is possible that the assay used to measure vibriocidal activity was not sensitive enough to detect such differences.

The *nirB* promoter was also able to drive the expression of *tcfA* from *B. pertussis* in IEM101. Tcf was processed and exposed on the IEM101 surface, although only a low proportion of the population was highly positive in the FACScan assay. This could be due to the low level of Tcf production; alternatively, Tcf could have a different conformation or exposure on the *V. cholerae* surface, by interaction with and/or sterical hindrance by outer membrane structures. However, it should be noted that only 31% of the *B. pertussis* population was recognized by the anti-MalE-Tcf antiserum used to probe Tcf. This could mean that the antibodies directed against the MalE-Tcf fusion protein do not bind efficiently to the Tcf protein in its native form.

Mouse immunization with *Vibrio* bacteria expressing Tcf resulted in a protective effect against *Bordetella* infection at the tracheal level, with approximately 10-fold reduction in colonization. These results corroborate previous observations that *tcf* mutants are less able to colonize the mouse trachea after aerosol infection than is the wild-type strain (15). The nature of the protective immunity induced by *Vibrio*-delivered Tcf is not known, as we were not able to detect a specific anti-Tcf immune response in the sera of immunized mice. Tcf shares an RGD motif with filamentous hemagglutinin and pertactin. The RGD motifs from both filamentous hemagglutinin (20) and pertactin (23) have been shown to be involved in adherence to host cells. Therefore, antibodies against Tcf could provide protection, since they could bind to Tcf on the *Bordetella* surface, preventing efficient bacterial adhesion to trachea mucosa; in addition, they could have bactericidal or opsonic activity. On the other hand, since cell-mediated responses play an important role in protection against *Bordetella* infection in the murine model (27, 32), the possibility that *V. cholerae*-delivered Tcf induced cellular responses should also be considered.

We did not observe protection at the lung level, and this result agrees with the observation that *tcf* mutants present the same pattern of lung colonization after aerosol infection as does wild-type *Bordetella* (15). Both observations could be due to lack of Tcf expression at that particular environment by *Bordetella* and/or the expression of other adhesion factors with more relevant roles in lung colonization.

In conclusion, we demonstrated the potential of *V. cholerae* as a delivery system for heterologous antigens, since two model antigens from nonenteropathogens were delivered by live *Vibrio*, and protective responses were elicited in both cases. We also showed that mouse intranasal inoculation with *V. cholerae* can be used for preliminary evaluation of immunogenicity. In order to enhance the immunogenicity of heterologous product, improvements in expression level and stability should be achieved by investigating different promoters; a promising alternative is the use of promoters from in vivo-induced (*ivi*) genes (7).

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