Transcutaneous Immunization with Cross-Reacting Material CRM₁₉₇ of Diphtheria Toxin Boosts Functional Antibody Levels in Mice Primed Parenterally with Adsorbed Diphtheria Toxoid Vaccine[∇]

Paul Stickings,¹ Marisa Peyre,¹ Laura Coombes,¹ Sylviane Muller,² Rino Rappuoli,³ Giuseppe Del Giudice,³ Charalambos D. Partidos,^{2†} and Dorothea Sesardic^{1*}

Division of Bacteriology, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Hertfordshire EN6 3QG, United Kingdom¹; UPR 9021, Institut de Biologie Moléculaire et Cellulaire, CNRS, 15 rue René Descartes, 67084 Strasbourg, France²; and Novartis Vaccines, Via Fiorentina 1, 53100 Siena, Italy³

Received 11 June 2007/Returned for modification 5 September 2007/Accepted 22 January 2008

Transcutaneous immunization (TCI) capitalizes on the accessibility and immunocompetence of the skin, elicits protective immunity, simplifies vaccine delivery, and may be particularly advantageous when frequent boosting is required. In this study we examined the potential of TCI to boost preexisting immune responses to diphtheria in mice. The cross-reacting material (CRM₁₉₇) of diphtheria toxin was used as the boosting antigen and was administered alone or together with either one of two commonly used mucosal adjuvants, cholera toxin (CT) and a partially detoxified mutant of heat-labile enterotoxin of *Escherichia coli* (LTR72). We report that TCI with CRM₁₉₇ significantly boosted preexisting immune responses elicited after parenteral priming with aluminum hydroxide-adsorbed diphtheria toxoid (DTxd) vaccine. In the presence of LTR72 as an adjuvant, toxin-neutralizing antibody titers were significantly higher than those elicited by CRM₁₉₇ alone and were comparable to the functional antibody levels induced after parenteral booster immunization with the adsorbed DTxd vaccine. Time course study showed that high levels of toxin-neutralizing antibodies persisted for at least 14 weeks after the transcutaneous boost. In addition, TCI resulted in a vigorous antigen-specific proliferative response in all groups of mice boosted with the CRM₁₉₇ protein. These findings highlight the promising prospect of using booster administrations of CRM₁₉₇ via the transcutaneous route to establish good herd immunity against diphtheria.

Diphtheria is an acute, often fatal bacterial disease caused by *Corynebacterium diphtheriae*. Clinical manifestations of the disease are due mainly to the presence of circulating diphtheria toxin (DTx), and immunity against diphtheria is due primarily to the presence of circulating antitoxin neutralizing antibodies. Active immunization programs against diphtheria are based on the use of a formaldehyde-detoxified preparation of DTx (diphtheria toxoid [DTxd]) to induce protective antibody responses, and these vaccination programs have had a remarkable effect in reducing the incidence of disease in developed countries. However, antidiphtheria antibody levels decrease with increasing age, and frequent booster vaccinations are required to maintain herd immunity in the adult population (5, 14, 18). The importance of maintaining high levels of seroprotection against diphtheria has been highlighted following the resurgence of disease in several European countries where a high proportion of the adult population were found to have antidiphtheria antibody titers below the putative protective levels of 0.01 IU/ml (4, 16).

DTxd vaccines have been associated with adverse reactions, particularly following booster immunization in adults (5, 26). The frequency of reactions is determined by a number of factors, including the degree of toxin purification prior to formaldehyde detoxification, the dose of antigen in the vaccine, and the immune status of the vaccine recipient (5). This is complicated by the fact that diphtheria booster immunizations are often administered in the form of divalent diphtheria-tetanus vaccines, and the presence of high levels of circulating tetanus antitoxin may also contribute to adverse reactions to the booster dose (5). Toxoid vaccines are also commonly adsorbed onto aluminum salts which act as an adjuvant, and the presence of these salts may contribute to some of the side effects observed (17). For specifically boosting immunity to diphtheria in susceptible populations, the availability of an antigen preparation with less adverse effects than those associated with conventional vaccines, combined with simple, practical, and noninvasive delivery, may lead to better disease control by increasing compliance and convenience of booster immunizations.

The cross-reacting material (CRM₁₉₇) of diphtheria toxin is a genetically detoxified preparation of the toxin (6, 13). This mutant of DTx does not require detoxification with formaldehyde, and homogeneous preparations of purified antigen can be readily obtained (26). CRM₁₉₇ is licensed for human use as a carrier protein for several capsular polysaccharide antigens

* Corresponding author. Mailing address: Division of Bacteriology, NIBSC, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, United Kingdom. Phone: 44 (0) 1707 641447. Fax: 44 (0) 1707 641054. E-mail: dsesardic@nibsc.ac.uk.

† Present address: Axion Analytical Laboratories, 14 North Peoria St., Chicago, IL.

[∇] Published ahead of print on 28 January 2008.

and is a promising vaccine candidate and potential alternative to conventional DTxd vaccines, particularly as a boosting antigen. Parenteral administration of CRM₁₉₇ would require needles, syringes, and trained medical personnel. In addition, soluble antigens such as CRM₁₉₇ are more susceptible to proteolytic degradation and are less immunogenic than conventional toxoid vaccines when administered parenterally (13). Alternative strategies for immunization against diphtheria with CRM₁₉₇ include delivery via the mucosal or transcutaneous route. Native CRM₁₉₇ is a poor antigen when delivered mucosally, although it has been shown to be an effective mucosal vaccine delivered intranasally in mice when conjugated to starch microparticles (27) or formulated with chitosan (20). In addition, CRM₁₉₇ formulated with chitosan was also found to be well tolerated in humans and stimulated humoral and cellular responses following intranasal immunization (21, 22).

Transcutaneous immunization (TCI) capitalizes on the accessibility and immunocompetence of the skin (8) and, like mucosal vaccination, minimizes the possibility of adverse reaction to antigen. This noninvasive immunization procedure elicits protective immunity (15, 19, 33), simplifies vaccine delivery, and may be particularly advantageous when frequent boosting is required, as in the case of diphtheria. TCI may also be more suitable for use with native CRM₁₉₇, removing the requirement for additional stabilization/conjugation of the protein that is required for optimum immune responses when CRM₁₉₇ is administered via the intranasal route (20). CRM₁₉₇ has previously been shown to induce anti-diphtheria toxin neutralizing antibodies in mice when delivered transcutaneously, although only in the presence of cholera toxin (CT) as an adjuvant (10). In this study we have examined the potential of TCI with native CRM₁₉₇ alone or together with either one of two commonly used mucosal adjuvants to efficiently boost preexisting immune responses to diphtheria elicited by priming parenterally with a WHO International Standard aluminum hydroxide-adsorbed DTxd vaccine. In addition, we assessed and compared the adjuvant effect of CT and LTR72, a partially detoxified mutant of heat-labile enterotoxin of *Escherichia coli* (LT) on the induction of anti-diphtheria toxin neutralizing antibody levels with those induced by boosting with adsorbed DTxd vaccine given by the subcutaneous (s.c.) route.

MATERIALS AND METHODS

Immunization procedures. For parenteral priming, we used the WHO Third International Standard for DTxd (adsorbed) vaccine (NIBSC 98/560, with defined activity of 160 IU per ampoule) (29). The vaccine was reconstituted in sterile 0.9% sodium chloride prior to administration. All groups of mice (female BALB/c mice, 6 to 8 weeks old, seven per group) were injected s.c. with 0.5 ml of the stock preparation containing 5 IU/ml adsorbed DTxd vaccine (2.5 IU/dose). Twelve weeks after priming, groups of mice were boosted s.c. with adsorbed DTxd vaccine or via the transcutaneous route with native CRM₁₉₇ (Novartis Vaccines, Siena, Italy) alone or with CT (Sigma, St. Louis, MO) or LTR72 (Novartis Vaccines, Siena, Italy) as an adjuvant. For TCI, the skin of a small surface area of the abdomen (approximately 2.5 cm²) was mildly ablated using a razor (no cuts were observed), and the hair was removed completely following application of a depilatory cream (Nair) for 1 to 2 min. The cream was completely removed using cotton wool soaked in lukewarm water, and the skin surface was swabbed with 70% ethanol. The prepared skin surface was then hydrated for 5 minutes using sterile phosphate-buffered saline (PBS) prior to application of antigen. The treated surface of the skin was blotted dry, and 50 µl of antigen solution containing combinations of CRM₁₉₇ (10 µg/dose), CT (20 µg/dose), and LTR72 (20 µg/dose) in PBS were applied topically. An additional

control group received a topical application of PBS vehicle alone. During TCI procedures, mice were anesthetized by an intraperitoneal injection of 0.15 ml of ketamine (100 mg/ml) and xylazine (2% [vol/vol]) in 0.9% sodium chloride and were immobilized for approximately 1 h to allow for antigen absorption and prevent possible mucosal uptake of antigen solutions. At the end of the immunization procedure, topically applied antigen was removed by blotting with a tissue followed by washing with tepid water.

ELISA for measurement of antibody responses. To measure the total anti-CRM₁₉₇ and anti-DTxd immunoglobulin G (IgG) antibody responses, Nunc Maxisorb 96-well enzyme-linked immunosorbent assay (ELISA) plates were coated with 100 µl of CRM₁₉₇ antigen (1.35 µg/ml) or nonadsorbed DTxd (NIBSC 02/176, 0.5 flocculation unit/ml) per well. Coating antigens were diluted in carbonate buffer (pH 9.6), and antigen-coated plates were incubated overnight at 4°C. The ELISA plates were then washed in PBS containing 0.05% (vol/vol) Tween 20 (PBS-T) and blocked with 150 µl of PBS-T containing 5% (wt/vol) skim milk powder (Marvel) for 1 h at 37°C. Following a second wash in PBS-T, serial dilutions of individual mouse serum samples (diluted in PBS-T containing 1% [wt/vol] skim milk powder) were prepared and placed in wells across the plate, and the plates were incubated at 37°C for 2 h. Plates were washed as described previously, and antigen-specific IgG antibodies were detected using a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (catalog no. A-9044; Sigma) diluted 1:2,000 in PBS-T containing 1% (wt/vol) skim milk powder. After a further 1-h incubation at 37°C and a final wash, the chromogen solution ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] (catalog no. A-9941; Sigma) in 0.05 M phosphate-citrate buffer (pH 4.0) was added, and the reaction was allowed to develop for 30 min. The optical density was measured at 405 nm (*A*₄₀₅) by a Multiscan ELISA plate reader (ThermoLifeSciences, United Kingdom). Antibody responses were analyzed by an in-house parallel-line bioassay program and were expressed as titers (for CRM₁₉₇-coated plates) or in international units/milliliter (for DTxd-coated plates) against an in-house mouse reference serum (0.12 IU/ml).

For measurement of IgG1 and IgG2a subclasses, an ELISA protocol similar to that described above was followed, and ELISA plates were coated with CRM₁₉₇ or DTxd as described above. Reference anti-mouse IgG1 and anti-mouse IgG2a antibodies (Sigma, Dorset, United Kingdom) were diluted 1:4,000 (0.25 µg/ml) in PBS-T containing 1% (wt/vol) skim milk powder. To detect specific binding, biotin anti-mouse IgG1 or IgG2a antibodies (BD Biosciences, United Kingdom) were used at a dilution of 1:5,000 (0.1 µg/ml). ExtrAvidin peroxidase conjugate (catalog no. E-2886; Sigma) diluted 1:1,000 was then added for 1 h at 37°C prior to the addition of ABTS substrate solution as described above. The presence of antigen-specific IgG1 and IgG2a antibodies was detected and measured as described above. The results were analyzed by an in-house parallel-line bioassay program, and antibody levels were expressed in micrograms per milliliter.

Vero cell assay for detection of anti-DTx neutralizing antibodies. The neutralizing capacity of anti-DTx antibodies was measured using the Vero cell toxin neutralization assay as described in detail elsewhere (10, 30, 34). The neutralizing potency of individual mouse serum samples, expressed in international units per milliliter, was calculated relative to an in-house murine reference serum calibrated in international units for diphtheria antitoxin against the WHO International Standard for Diphtheria Antitoxin (10 IU/ml).

Measurement of proliferative T-cell responses. Spleens were aseptically removed 14 weeks after the booster immunization (26 weeks after priming), and a single-cell suspension was prepared by passing through a sterile cell strainer (BD Falcon, BD Biosciences, United Kingdom). After the cells were washed, they were resuspended in RPMI 1640 (Invitrogen, Paisley, United Kingdom) containing 10% (vol/vol) heat-inactivated fetal bovine serum (Invitrogen), 1% (vol/vol) L-glutamine (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B (Sigma), and 50 µM 2-mercaptoethanol (Sigma). Viable splenocytes (2 × 10⁵ cells per well) were cultured in complete culture medium in 96-well flat-bottomed microtiter plates (Falcon). For antigen restimulation, splenocytes were cultured in complete medium containing 0.1 to 10 µg/ml CRM₁₉₇ for transcutaneous groups receiving CRM₁₉₇ as the antigen (or PBS vehicle) or 0.1 to 10 µg/ml purified DTxd (6,000 flocculation units/ml) for the group boosted s.c. with adsorbed DTxd vaccine. Spleen cells from each group were also stimulated with 1 µg/ml concanavalin A (Sigma) as a positive control or cultured in complete medium alone as a negative control. Cells were cultured in humidified air containing 5% CO₂ at 37°C for 4 days. The cells were then pulsed for 8 h with 0.5 µCi/well [³H]thymidine (Amersham Biosciences, United Kingdom) and harvested onto glass-fiber filter mats (PerkinElmer LAS, United Kingdom) using a Micro 96 harvester (Molecular Devices, United Kingdom). Radioactivity incorporated into cellular DNA was measured by counting filter mats in a 1450 MicroBeta liquid scintillation counter (PerkinElmer LAS, United

Kingdom). Results were expressed as a stimulation index of the mean counts per minute obtained from triplicate cultures in the presence of specific antigen (CRM₁₉₇ or DTxd) divided by the mean counts per minute of triplicate cultures incubated in complete medium only. A stimulation index greater than 2 was considered positive. The mean stimulation index for the positive control was 71.3 ± 8.6 , and the mean background counts per minute in unstimulated splenocyte cultures from all five groups of mice was 773.9 ± 242.4 (not shown).

Cytokine ELISA. Spleen cell cultures prepared for proliferation measurement were also used to measure antigen-specific cytokine production. Viable splenocytes (2×10^6 cells per well) were cultured in 24-well tissue culture plates in medium alone or supplemented with CRM₁₉₇ or DTxd (0.1 to 10 μ g/ml). After 72 h, culture supernatants were transferred into 1.5-ml centrifuge tubes and centrifuged at $16,060 \times g$ for 5 min to remove nonadherent cells and debris. Cytokine concentrations in the cell supernatants were measured by sandwich ELISA using the appropriate commercial ELISA kits according to the manufacturer's instructions (BD Biosciences, United Kingdom). The results were expressed as the mean cytokine concentration (in picograms per milliliter) $\pm$ standard error of the mean (SEM) from triplicate cultures after extrapolation from a standard curve prepared with the reference cytokine supplied with each kit.

Statistical analysis. For comparison of results within experimental groups, a Student's *t* test was performed. For all multigroup comparisons, one-way analysis of variance (ANOVA) was used. Where significant differences were observed, Tukey's multiple comparison test was used to identify significant differences between individual groups.

RESULTS

Transcutaneous booster immunization using CRM₁₉₇ with and without adjuvant significantly increases systemic diphtheria antibody responses in mice. Parenteral (s.c.) priming with 2.5 IU/dose ($\sim 1/20$ th of human dose) of aluminum hydroxide-adsorbed DTxd vaccine elicited weak antibody responses in all five groups of mice. Ten weeks after priming, the mean antibody titer across all five groups was 0.56 ± 0.03 IU/ml. After 12 weeks, one group of mice received a second parental immunization with the same dose of adsorbed DTxd vaccine, and the anti-diphtheria IgG titers were significantly increased by 10-fold compared to preboost titers (6.08 ± 0.77 IU/ml; $P < 0.005$). Anti-diphtheria IgG levels remained high in this group and were not significantly different (14 weeks after the boost) compared to the responses measured 2 weeks after the boost (not shown).

Analysis of postboost serum antibody levels demonstrated a significant increase in anti-CRM₁₉₇ IgG antibody titers in all groups of mice that were boosted via the transcutaneous route with CRM₁₉₇. Two weeks after the TCI boost, anti-CRM₁₉₇ IgG titers were significantly increased in the group immunized with CRM₁₉₇ alone or with CRM₁₉₇ given together with CT or LTR72 as the adjuvant compared to preboost antibody titers in the same group (Fig. 1). ANOVA from time course studies demonstrated that at all time points postboost (up to 14 weeks postboost), all groups that were boosted with CRM₁₉₇ alone or with adjuvant had significantly higher anti-CRM₁₉₇ IgG titers than those of the PBS control group at the same time point (Fig. 1). The peak antibody response was observed 6 weeks postboost (week 18) in all three groups that were immunized with CRM₁₉₇ (with or without adjuvant), and there was no significant difference between the anti-CRM₁₉₇ antibody titers in the group immunized with CRM₁₉₇ alone and those in either the group that received CT or the group that received LTR72 as the adjuvant at any time point (Fig. 1).

FIG. 1. Serum anti-CRM₁₉₇ antibody responses elicited in mice following TCI boost using CRM₁₉₇ alone or with adjuvant. Mice in all groups were primed with adsorbed DTxd vaccine (2.5 IU/dose) and were test bled after 10 weeks to determine preboost anti-CRM₁₉₇ antibody titers. At week 12, mice were boosted via the transcutaneous route with CRM₁₉₇ alone or with adjuvant (indicated by the arrow). A control group received PBS vehicle alone. Test bleed samples were taken from all animals at 2, 6, 10, and 14 weeks after the boost and analyzed for specific anti-CRM₁₉₇ antibodies by ELISAs. Time (in weeks) is shown on the x axis. TCI boost using CRM₁₉₇ with adjuvant ($P < 0.01$) or without adjuvant ($P < 0.005$) significantly increased antibody responses measured 2 weeks after the boost compared to preboost titers in the same group (paired *t* test). ANOVA was performed for all groups at each time point after the boost (weeks 14, 18, 22, and 26) and detected significant differences at each time point ($P < 0.001$). At each time point after the boost, anti-CRM₁₉₇ antibody titers were significantly higher in all three vaccine groups than in the PBS control group at the same time point ($P < 0.001$). There was no significant difference in antibody titers between groups immunized using CRM₁₉₇ with or without adjuvant at any time point. Data are the means $\pm$ SEMs (error bars) from seven mice per group.

Transcutaneous booster immunization with CRM₁₉₇ and adjuvant induces levels of anti-diphtheria toxin neutralizing antibodies that are comparable to those induced by subcutaneous booster immunization using a conventional adsorbed DTxd vaccine. Functional toxin-neutralizing antibodies were measured in individual mouse serum samples from all groups using the Vero cell assay. This allows for direct comparison between groups that received different antigens via different immunization routes. Preboost neutralizing antibody titers were low in all groups following parenteral priming with the adsorbed DTxd vaccine, with a mean antibody titer of 0.12 ± 0.01 IU/ml for all five groups. Two weeks after s.c. boost with adsorbed DTxd vaccine, the neutralizing antibody titers in this group had increased 40-fold compared to the preboost titers (Fig. 2). TCI boost using CRM₁₉₇ with or without adjuvant also had a marked effect on the circulating anti-DTx neutralizing antibodies. Mice boosted with CRM₁₉₇ alone, CRM₁₉₇ plus CT, and CRM₁₉₇ plus LTR72 all showed significant increases in functional antibody levels compared to preboost titers for mice in the same group (Fig. 2). The effect of adjuvant on the booster immunization was also determined. At week 14 (2 weeks postboost), mice boosted with the adsorbed DTxd vaccine (s.c.) or with CRM₁₉₇ plus LTR72 (TCI) had significantly higher neutralizing antibody titers than the group immunized

FIG. 2. Anti-DT_x neutralizing antibodies elicited in mice following transcutaneous or parenteral boost. Mice were primed and boosted as described in the legend to Fig. 1. Another group of mice was boosted by a second parenteral immunization with the adsorbed DT_xd vaccine (2.5 IU/dose). Pre- and postboost serum samples were analyzed for anti-DT_x neutralizing antibodies using the Vero cell assay. Time (in weeks) is shown on the x axis. Neutralizing antibody titers were significantly increased by TCI boost using CRM₁₉₇ alone ($P < 0.005$) or with CT ($P < 0.005$) or LTR72 ($P < 0.05$) as the adjuvant compared to preboost titers in the same group (paired t test). Subcutaneous boost with the adsorbed DT_xd vaccine also increased neutralizing antibody responses compared to preboost titers ($P < 0.005$). ANOVA was performed for all four vaccine groups at each time point after the boost. At week 14 (2 weeks after the boost), neutralizing antibody titers were significantly higher in the group given LTR72 as the adjuvant ($P < 0.005$) and the group given adsorbed DT_xd ($P < 0.05$) than in mice immunized with CRM₁₉₇ alone ($P < 0.005$ by the main ANOVA). Six weeks after the boost, responses were significantly higher in the group given LTR72 as the adjuvant ($P < 0.05$) and in the group given adsorbed DT_xd ($P < 0.05$) than in mice boosted with CT as the adjuvant ($P < 0.005$ by the main ANOVA). At later time points (10 and 14 weeks postboost), there were no significant differences between any of the vaccine groups with or without adjuvant. Data are the means $\pm$ SEMs (error bars) from seven mice per group.

with CRM₁₉₇ alone (Fig. 2). However, transcutaneous boost using CRM₁₉₇ with CT as the adjuvant did not significantly increase the anti-DT_x response compared to the response observed with CRM₁₉₇ alone. For groups receiving antigen plus adjuvant, postboost neutralizing antibody responses peaked 2 weeks after the boost (week 14) and were highest in the group boosted transcutaneously using CRM₁₉₇ plus LTR72 (4.7 ± 1.3 IU/ml), although there was no significant difference between the responses induced by boosting with any of the three adjuvants (Fig. 2).

Long-term neutralizing antibody responses induced by TCI with CRM₁₉₇ alone are comparable to those induced using CRM₁₉₇ with adjuvant or parenteral boost with adsorbed DT_xd vaccine. Analysis of the kinetics of the functional antibody response revealed that neutralizing antibody titers decreased more rapidly over time in the three groups treated with adjuvant (CT, LTR72, and adsorbed DT_xd vaccine). Six weeks after the booster immunization (week 18), there was no significant difference in neutralizing antibody titers between the group not treated with adjuvant (CRM₁₉₇ alone) and the groups treated with adjuvant (Fig. 2). At this time point, mice boosted with LTR72 as the adjuvant or with the adsorbed DT_xd vaccine had significantly higher neutralizing antibody

titers than mice boosted with CT as the adjuvant (Fig. 2). By week 26 (14 weeks postboost), neutralizing antibody levels had fallen by 63% in the CRM₁₉₇-plus-CT-treated group ($P < 0.005$), 62% in the CRM₁₉₇-plus-LTR72-treated group ($P < 0.05$), and 65% in the alum-adsorbed DT_xd group ($P < 0.05$) compared to the peak response seen at week 14. In contrast, postboost antibody levels in the group immunized transcutaneously using native CRM₁₉₇ without adjuvant remained constant with only a 2% reduction in titers at week 26 compared to the maximum response seen at week 14, 2 weeks after the boost. At the end of the study (14 weeks after the boost), there was no significant difference in neutralizing antibody titers between the group boosted with CRM₁₉₇ alone and any of the groups treated with adjuvant (CT, LTR72, or adsorbed DT_xd). At this time, there were no significant differences between adjuvant or immunization route in terms of inducing a neutralizing antidiophtheria response (Fig. 2).

TCI adjuvant influences the IgG subclass response to immunization with CRM₁₉₇. In all groups of mice boosted via the transcutaneous route, the predominant IgG antibody subclass was IgG1, suggesting a Th2-type immune response (Fig. 3A and B). Parenteral boost with adsorbed DT_xd vaccine also induced antibodies that were predominantly of the IgG1 subclass. However, mice boosted using CRM₁₉₇ with CT as the adjuvant showed a significant reduction in the IgG1/IgG2a ratio compared to mice immunized with CRM₁₉₇ alone or with LTR72 as the adjuvant and compared to mice immunized parenterally with two doses of adsorbed DT_xd vaccine, indicating a shift toward a Th1-type immune response in the presence of this adjuvant (Fig. 3C).

TCI with CRM₁₉₇ induces potent antigen-specific T-cell responses. Antigen-specific T-cell responses following booster immunization with CRM₁₉₇ or adsorbed DT_xd vaccine were measured by the degree of splenocyte proliferation on restimulation with antigen *ex vivo*. Figure 4 shows that spleen cells from mice boosted transcutaneously with CRM₁₉₇ (with or without adjuvant) or subcutaneously with adsorbed DT_xd vaccine proliferated strongly when restimulated with antigen *in vitro*. As with the systemic antibody responses measured at the end of the study, there was no significant difference in splenocyte proliferation between those groups of mice boosted transcutaneously using CRM₁₉₇ with or without adjuvant. However, at the highest dose of restimulating antigen (10 μ g/ml), proliferation induced by immunization with the adsorbed DT_xd vaccine was significantly higher than that induced by transcutaneous boost with CRM₁₉₇ given alone or together with CT as adjuvant (Fig. 4). Antigen-specific cytokine production was also measured in cultured spleen cells restimulated with the appropriate antigen. Increased levels of gamma interferon (IFN- γ) were detected in the supernatants of spleen cells isolated from mice boosted transcutaneously with CRM₁₉₇ together with CT as the adjuvant (Table 1). This supports the IgG subclass findings and the suggestion that CT promotes a rather mixed Th1/Th2 immune response.

DISCUSSION

Successful pediatric vaccination programs against diphtheria have resulted in high levels of immunity in this age group associated with a decline in the incidence of disease and a

FIG. 3. IgG1 and IgG2a subclasses of antibodies elicited after transcutaneous boost with CRM₁₉₇ given with or without adjuvant or subcutaneous boost with adsorbed DTxd vaccine. Data are presented as individual anti-CRM₁₉₇ or anti-DTxd IgG1 (A) and anti-CRM₁₉₇ or anti-DTxd IgG2a (B) responses from all seven mice in each group with the geometric mean titer indicated by a solid line. (C) Ratio of anti-CRM₁₉₇ IgG1/IgG2a antibodies in each group. The antibody ratio was obtained from the respective IgG1 and IgG2a titers from individual mice, and data are expressed as the mean ratio plus SEM (error bar) from the seven mice in each group. Values that were significantly different from those of the group immunized with CRM₁₉₇ plus CT (ANOVA with Tukey's test, main P value < 0.005) are indicated (*, $P < 0.05$; ***, $P < 0.005$).

reduction in the reservoir of toxigenic *C. diphtheriae*. However, repeated booster immunizations against diphtheria are required to maintain immunity in the adult population. Despite formulation of reduced antigen, lower potency products for this purpose, local and systemic reactions to the booster dose are observed in some adult recipients (5, 26), and antigen preparations, such as the nontoxic CRM₁₉₇, may offer a suitable alternative to the toxoid vaccines that have been associated with local adverse reactions in adults. We have investigated the use of native CRM₁₉₇ as a booster antigen for diphtheria vaccination via the transcutaneous route in mice.

Our findings demonstrated that CRM₁₉₇ was immunogenic and significantly boosted preexisting immune responses to diphtheria in mice when applied to bare skin alone or together

with the mucosal adjuvant CT or LTR72. Anti-CRM₁₉₇ antibody responses in mice were comparable in groups treated with adjuvant and groups not treated with adjuvant at all time points, highlighting the immunogenic potential of CRM₁₉₇ when delivered transcutaneously. The results from this study suggest that the physicochemical characteristics of CRM₁₉₇ are compatible for percutaneous penetration at sufficient concentrations to trigger an accelerated memory antibody response. This is consistent with published data demonstrating the immunogenic potential of CRM₁₉₇ when administered transcutaneously (10). Our data are also in agreement with published observations highlighting the suitability of CRM₁₉₇ as an antigen of choice for booster administrations via the intranasal route (20).

Analysis of functional neutralizing antibody levels using the

FIG. 4. Proliferative responses of splenocytes from mice boosted transcutaneously using CRM₁₉₇ with or without adjuvant or parenterally with adsorbed DTxd vaccine. Splenocytes isolated from the transcutaneous groups at the end of the study (week 26) were restimulated in the presence of 0.1, 1, or 10 µg/ml CRM₁₉₇. Splenocytes isolated from the group primed and boosted parenterally with adsorbed DTxd vaccine were restimulated with the same concentrations of purified DTxd. Data are the means plus SEMs (error bars) from three separate spleen cell cultures. A stimulation index of >2 (indicated by a solid line) was considered positive. ANOVA detected significant differences between the four vaccine groups at the highest dose of restimulating antigen only (ANOVA with Tukey's test, main *P* value < 0.005). Values that were significantly different from the value of the group treated with CRM₁₉₇ plus CT (*, *P* < 0.05) and the value of the group treated with CRM₁₉₇ (##, *P* < 0.01) at the same dose of restimulating antigen are shown.

Vero cell assay allows for direct comparison between groups that received different antigens via different immunization routes. We have shown that the antibodies induced by transcutaneous boost with CRM₁₉₇ had high neutralizing capacity against DTx (>1 IU/ml). Parenteral boost with adsorbed DTxd vaccine also induced high levels of toxin-neutralizing antibodies, and the postboost response was significantly higher than that induced following TCI boost with CRM₁₉₇ alone. The inclusion of CT as the adjuvant for TCI boost did not significantly increase functional antibody responses compared to boosting with CRM₁₉₇ alone. However, the presence of LTR72 as the adjuvant significantly increased functional antibody levels compared to TCI boost with CRM₁₉₇ alone (Fig. 2). This suggests that LTR72 was able to significantly affect the quality of the immune response by increasing the levels of functional toxin-neutralizing antibodies, even though total anti-CRM₁₉₇ titers were comparable between the groups boosted by TCI with and without adjuvant (Fig. 1).

By monitoring the kinetics of the neutralizing antibody responses, it was interesting to note that antibody levels declined faster over time in those animals where CRM₁₉₇ was given together with adjuvant compared to the response to immunization with CRM₁₉₇ alone. A similar drop in functional antibody titers over time was also seen in the group primed and boosted with the aluminum hydroxide-adsorbed DTxd vaccine.

TABLE 1. Cytokine production from splenocytes of mice boosted via the TCI and s.c. routes^a

Treatment group	Level of IFN-γ produced (pg/ml) with the following concn of restimulating antigen (µg/ml):				Level of IL-5 ^b produced (pg/ml) with the following concn of restimulating antigen (µg/ml):				Level of IL-4 produced (pg/ml) with the following concn of restimulating antigen (µg/ml):			
	0	0.1	1	10	0	0.1	1	10	0	0.1	1	10
CRM ₁₉₇	5.6 ± 3.8	20.1 ± 12.4	29.5 ± 19.2	119.0 ± 81.1	5.9 ± 5.9	7.5 ± 7.5	37.4 ± 12.8	121.3 ± 37.5	0.7 ± 0.5	2.5 ± 2.3	3.7 ± 3.4	12.3 ± 7.2
CRM ₁₉₇ + CT	79.7 ± 45.9	689.4 ± 483.9	932.2 ± 505.0	929.1 ± 344.4	0.5 ± 0.5	35.6 ± 13.9	60.6 ± 21.5	136.0 ± 24.6	8.6 ± 4.8	40.0 ± 23.9	52.8 ± 25.6	56.2 ± 22.8
CRM ₁₉₇ + LTR72	2.9 ± 1.4	80.0 ± 20.2	208.3 ± 37.6	405.5 ± 43.4	0.0 ± 0.0	13.5 ± 6.8	52.5 ± 18.9	148.7 ± 44.0	0.0 ± 0.0	5.9 ± 1.3	15.7 ± 2.8	28.3 ± 8.0
PBS (control)	2.3 ± 2.2	8.6 ± 6.4	12.6 ± 10.8	48.8 ± 27.2	0.0 ± 0.0	2.8 ± 2.8	19.2 ± 19.3	41.0 ± 41.1	0.0 ± 0.0	0.8 ± 0.8	0.7 ± 0.7	3.3 ± 3.3
DTxd (s.c.)	7.3 ± 5.5	39.2 ± 20.8	73.9 ± 47.2	354.3 ± 203.4	12.3 ± 12.3	75.2 ± 53.8	133.1 ± 83.3	317.6 ± 132.3	0.6 ± 0.6	5.0 ± 2.9	8.9 ± 5.7	22.8 ± 11.5

^a Spleen cells isolated from the four groups boosted by TCI were restimulated in the presence of 0.1 to 10 µg/ml CRM₁₉₇. Splenocytes isolated from the group boosted parenterally with DTxd vaccine were restimulated in the presence of 0.1 to 10 µg/ml of purified DTxd. ANOVA was performed for all three cytokines at each dose of restimulating antigen. At the highest dose of restimulating antigen, TCI boost with CRM₁₉₇ with CT as the adjuvant significantly increased splenocyte production of IFN-γ compared to cells isolated from mice in the PBS control group (*P* < 0.05). Data are the means ± SEMs from three separate spleen cell cultures.

^b IL-5, interleukin 5.

The response to TCI boost with CT fell sharply 6 weeks after the boost, and at this time point (week 18), both the LTR72 (TCI) and alum (s.c.) adjuvants induced significantly higher functional responses compared to the responses in mice boosted with CT as the adjuvant (Fig. 2). Neutralizing antibody responses measured at the end of the study, 14 weeks after boosting, were comparable between groups boosted using CRM₁₉₇ alone or with adjuvant (CT, LTR72, or DTxd). This suggests that although adjuvant may affect the quality of the immune response in the short term following booster vaccination, long-term and sustained protective immunity induced by TCI boost may not require powerful adjuvants. However, further studies with extended bleeding times after boosting are required to support this conclusion.

TCI is a procedure that elicits potent antigen-specific proliferative T-cell responses (1, 10, 11). In this study we observed the induction of strong proliferative responses after TCI boost with CRM₁₉₇. Cellular responses did not correlate in all cases with the antibody responses from the same time point, and at the highest dose of restimulating antigen, proliferative responses from mice boosted with the adsorbed DTxd vaccine were higher than those from mice boosted with CRM₁₉₇ alone or with CT as the adjuvant. The data obtained from the subclass profile of circulating antibodies suggest that the immune responses were mainly of the Th2 type. However, transcutaneous boost using CT as the adjuvant caused a significant reduction in the IgG1/IgG2a ratio and increased levels of IFN- γ production, suggesting that this adjuvant promoted a more mixed Th1/Th2 response. This is consistent with previous data obtained using CRM₁₉₇ together with CT as the adjuvant via the transcutaneous route (10). Unlike CT, the LTR72 adjuvant used in this study did not skew the immune response, although this adjuvant has been shown to induce strong cellular immunity and mixed Th1/Th2 responses to meningococcal B proteins delivered intranasally in mice (2). This suggests that the nature of the immune response is influenced not only by antigen and adjuvant combinations but also by the route of immunization. However, a skewed Th1/Th2 immune response is not a critical factor for protection against diphtheria where clinical symptoms are almost entirely due to the presence of circulating toxin, and the type of immune response observed in this study did not appear to have any significant effect on the neutralizing capacity of circulating antibodies.

The precise molecular mechanisms of adjuvanticity of CT and LTR72 after TCI are not fully understood. While the holotoxin CT retains ADP-ribosylation activity, the *Escherichia coli* heat-labile enterotoxin LT mutant (LTR72) has only 0.6% of the enzymatic activity of wild-type LT (7). However, we show that the toxin mutant LTR72 used in this study significantly enhances postboost functional antibody levels, an effect that was not seen using the fully enzymatically active CT holotoxin. This suggests that full ADP-ribosylating activity is not required for significant enhancement of the immune response to a topically coadministered antigen and is consistent with previous findings where several adjuvants with no ADP-ribosylating activity were shown to enhance antibody responses to a topically coapplied diphtheria toxoid (28). However, the presence of some enzymatic activity appears to be important for optimum adjuvanticity, and the spiking of a recombinant CTB subunit (devoid of enzyme activity) with a small amount

of holotoxin was shown to induce immune responses that were comparable to those obtained with the native CT (28). LTR72 which retains residual enzymatic activity is a more effective mucosal adjuvant than the related LT derivative LTK63 which is devoid of enzymatic activity (7). Our data show that a mutant toxin with residual enzymatic activity is a powerful transcutaneous adjuvant and induces stronger functional immune responses than a fully active holotoxin in the short term after the boost. Although we did not compare the adjuvant effect of LTR72 and the wild-type LT holotoxin in this study, CT and LT are functionally, structurally, and immunologically similar (32), and consistent with our own findings, LTR72 has been shown to be a more effective adjuvant than wild-type LT in TCI (25) and intranasal immunization (2).

The choice of adjuvant for transcutaneous immunization will depend not only on the strength and quality of the enhanced immune response but also on the safety of the adjuvant in question. The inherent toxicity of bacterial toxins, such as CT and LT, raises specific concerns with their use as adjuvants in human vaccines. This is particularly true for mucosal immunization where CT and LT have been shown to undergo retrograde transport along olfactory nerves to olfactory bulbs in the brains of mice immunized via the intranasal route (reviewed in reference 3). In humans, severe adverse reactions including Bell's palsy have been reported following intranasal immunization with LT (24, 31). These concerns are perhaps less relevant for skin delivery and are likely to be site and route specific due to the proximity of key neurological pathways to the site of antigen/adjuvant application at the nasal mucosa. The LT holotoxin is reportedly safe when used as an adjuvant on human skin (9, 12). However, this toxin has been shown to cause some mild local side effects following TCI in humans (reviewed in reference 31), and partially or fully detoxified mutants of bacterial toxins, such as LTR72, may become adjuvants of choice for future human use—particularly when they are shown to be equally or more effective as fully active holotoxins. However, further studies are required in humans using the transcutaneous route to determine the safety profile and efficacy of these new adjuvants.

Despite the fact that mice lack the required receptors for DTx binding, which significantly reduces the immunogenicity of CRM₁₉₇ in this model (13, 23), we have shown that topical application of CRM₁₉₇, without additional stabilization or formulation, is highly immunogenic for boosting diphtheria immunity in mice after parenteral priming with a conventional toxoid vaccine. Although initial neutralizing antibody responses were significantly enhanced in the presence of a mucosal adjuvant (LTR72), longer-term immunity induced by TCI using CRM₁₉₇ alone was comparable to immunity induced by TCI with adjuvant and to boosting with a conventional toxoid vaccine given by the classical parenteral route. This study highlights the suitability of CRM₁₉₇ and suggests that the native protein is sufficiently stable and immunogenic for transcutaneous booster immunization against diphtheria. Our findings highlight the potential of TCI as an alternative and effective immunization route for boosting the waning levels of functional toxin-neutralizing antibodies in the adult population.

ACKNOWLEDGMENTS

This work was financed in part by the Centre National de la Recherche Scientifique (CNRS) and the National Institute for Biological Standards and Control (NIBSC).

R.R. and G.D.G. are full-time employees of Novartis Vaccines and Diagnostics.

We thank technicians at NIBSC for excellent animal husbandry and assistance with TCI procedures and Peter Rigsby at NIBSC for help with statistical analysis.

REFERENCES

1. Beignon, A. S., J. P. Briand, R. Rappuoli, S. Muller, and C. D. Partidos. 2002. The LTR72 mutant of heat-labile enterotoxin of *Escherichia coli* enhances the ability of peptide antigens to elicit CD4⁺ T cells and secrete gamma interferon after coapplication onto bare skin. *Infect. Immun.* **70**: 3012–3019.
2. Bowe, F., E. C. Lavelle, E. A. McNeela, C. Hale, S. Clare, B. Arico, M. M. Giuliani, A. Rae, A. Huett, R. Rappuoli, G. Dougan, and K. H. G. Mills. 2004. Mucosal vaccination against serogroup B meningococci: induction of bactericidal antibodies and cellular immunity following intranasal immunization with NadA of *Neisseria meningitidis* and mutants of *Escherichia coli* heat-labile enterotoxin. *Infect. Immun.* **72**:4052–4060.
3. Connell, T. D. 2007. Cholera toxin, LT-I, LT-IIa and LT-IIb: the critical role of ganglioside binding in immunomodulation by type I and type II heat-labile enterotoxins. *Expert Rev. Vaccines* **6**:821–834.
4. Galazka, A. 2000. Implications of the diphtheria epidemic in the Former Soviet Union for immunization programmes. *J. Infect. Dis.* **181**(Suppl. 1): S244–S248.
5. Galazka, A. M., and S. E. Robertson. 1996. Immunization against diphtheria with special emphasis on immunization of adults. *Vaccine* **14**:845–857.
6. Giannini, G., R. Rappuoli, and G. Ratti. 1984. The amino-acid sequence of two non-toxic mutants of diphtheria toxin: CRM₄₅ and CRM₁₉₇. *Nucleic Acids Res.* **12**:4063–4069.
7. Giuliani, M. M., G. Del Giudice, V. Gianelli, G. Dougan, G. Douce, R. Rappuoli, and M. Pizza. 1998. Mucosal adjuvanticity and immunogenicity of LTR72, a novel mutant of *Escherichia coli* heat-labile enterotoxin with partial knockout of ADP-ribosyltransferase activity. *J. Exp. Med.* **187**:1123–1132.
8. Glenn, G. M., R. T. Kenney, L. R. Ellingsworth, S. A. Frech, S. A. Hammond, and J. P. Zoetewij. 2003. Transcutaneous immunization and immunostimulant strategies: capitalizing on the immunocompetence of the skin. *Expert Rev. Vaccines* **2**:253–267.
9. Glenn, G. M., D. C. Flyer, L. R. Ellingsworth, S. A. Frech, D. M. Frerichs, R. C. Seid, Jr., and J. Yu. 2007. Transcutaneous immunization with heat-labile enterotoxin: development of a needle-free vaccine patch. *Expert Rev. Vaccines* **6**:809–819.
10. Godefroy, S., M. Peyre, N. Garcia, S. Muller, D. Sesardic, and C. D. Partidos. 2005. Effect of skin barrier disruption on immune responses to topically applied cross-reacting material (CRM₁₉₇) of diphtheria toxin. *Infect. Immun.* **73**:4803–4809.
11. Guebre-Xabier, M., S. A. Hammond, D. E. Epperson, J. Yu, L. Ellingsworth, and G. M. Glenn. 2003. Immunostimulant patch containing heat-labile enterotoxin from *Escherichia coli* enhances immune responses to injected influenza virus vaccine through activation of skin dendritic cells. *J. Virol.* **77**:5218–5225.
12. Güereña-Burgueño, F., E. R. Hall, D. N. Taylor, F. J. Cassels, D. A. Scott, M. K. Wolf, Z. J. Roberts, G. V. Nesterova, C. R. Alving, and G. M. Glenn. 2002. Safety and immunogenicity of a prototype enterotoxigenic *Escherichia coli* vaccine administered transcutaneously. *Infect. Immun.* **70**:1874–1880.
13. Gupta, R. K., R. J. Collier, R. Rappuoli, and G. R. Siber. 1997. Differences in the immunogenicity of native and formalinized cross reacting material (CRM₁₉₇) of diphtheria toxin in mice and guinea pigs and their implications on the development and control of diphtheria vaccine based on CRMs. *Vaccine* **15**:1341–1343.
14. Kaml, M., I. Weiskirchner, M. Keller, T. Luft, E. Hoster, J. Hasford, L. Young, B. Bartlett, C. Neuner, K. H. Fischer, B. Neuman, R. Würzner, and B. Grubeck-Loebenstein. 2006. Booster vaccination in the elderly: their success depends on the vaccine type applied earlier in life as well as on pre-vaccination antibody titers. *Vaccine* **24**:6808–6811.
15. Kenney, R. T., J. Yu, M. Guebre-Xabier, S. A. Frech, A. Lambert, B. A. Heller, L. R. Ellingsworth, J. E. Eyles, E. D. Williamson, and G. M. Glenn. 2004. Induction of protective immunity against lethal anthrax challenge with a patch. *J. Infect. Dis.* **190**:774–782.
16. Lai, S., and A. Efstratiou on behalf of the European Laboratory Working Group on Diphtheria. 2002. Report on the Sixth International Meeting of the European Laboratory Working Group on Diphtheria, Brussels, Belgium. *Eurosurveillance* **7**:7–12.
17. Lindblad, E. B. 2004. Aluminum adjuvants—in retrospect and prospect. *Vaccine* **22**:3658–3668.
18. Maple, P. A., A. Efstratiou, R. C. George, N. J. Andrews, and D. Sesardic. 1995. Diphtheria immunity in UK blood donors. *Lancet* **345**:963–965.
19. Mawas, F., M. Peyre, A. S. Beignon, L. Frost, G. Del Giudice, R. Rappuoli, S. Muller, D. Sesardic, and C. D. Partidos. 2004. Successful induction of protective antibody responses against *Haemophilus influenzae* type b and diphtheria after transcutaneous immunization with the glycoconjugate polyribosyl ribitol phosphate-cross-reacting material 197 vaccine. *J. Infect. Dis.* **190**:1177–1182.
20. McNeela, E. A., D. O'Connor, I. Jabbal-Gill, L. Illum, S. S. Davis, M. Pizza, S. Peppoloni, R. Rappuoli, and K. H. Mills. 2000. A mucosal vaccine against diphtheria: formulation of cross reacting material (CRM₁₉₇) of diphtheria toxin with chitosan enhances local and systemic antibody and Th2 responses following nasal delivery. *Vaccine* **19**:1188–1198.
21. McNeela, E. A., I. Jabbal-Gill, L. Illum, M. Pizza, R. Rappuoli, A. Podda, D. J. Lewis, and K. H. Mills. 2004. Intranasal immunization with genetically detoxified diphtheria toxin induces T cell responses in humans: enhancement of Th2 responses and toxin-neutralizing antibodies by formulation with chitosan. *Vaccine* **22**:909–914.
22. Mills, K. H., C. Cosgrove, E. A. McNeela, A. Sexton, R. Giemza, I. Jabbal-Gill, A. Church, W. Lin, L. Illum, A. Podda, R. Rappuoli, M. Pizza, G. E. Griffin, and D. J. Lewis. 2003. Protective levels of diphtheria-neutralizing antibody induced in healthy volunteers by unilateral priming-boosting intranasal immunization associated with restricted ipsilateral mucosal secretory immunoglobulin A. *Infect. Immun.* **71**:726–732.
23. Mitamura, T., S. Higahiyama, N. Taniguchi, M. Klagsbrun, and E. Mekada. 1995. Diphtheria toxin binds to the epidermal growth factor (EGF)-like domain of human heparin-binding EGF-like growth factor/diphtheria toxin receptor and inhibits specifically its mitogenic activity. *J. Biol. Chem.* **270**: 1015–1019.
24. Mutsch, M., W. Zhou, P. Rhodes, M. Bopp, R. T. Chen, T. Linder, C. Spyr, and R. Steffen. 2004. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *N. Engl. J. Med.* **350**:896–903.
25. Partidos, C. D., A.-S. Beignon, J.-P. Briand, and S. Muller. 2004. Modulation of immune responses with transcutaneously deliverable adjuvants. *Vaccine* **22**:2385–2390.
26. Rappuoli, R. 1997. New and improved vaccines against diphtheria and tetanus, p. 417–436. *In* M. M. Levine, M. C. Woodrow, J. B. Kaper, and G. S. Cobon (ed.), *New generation vaccines*, 2nd ed. Marcel Dekker, New York, NY.
27. Rydell, N., and I. Sjöholm. 2005. Mucosal vaccination against diphtheria using starch microparticles as adjuvant for cross-reacting material (CRM₁₉₇) of diphtheria toxin. *Vaccine* **23**:2775–2783.
28. Scharton-Kersten, T., J.-M. Yu, R. Vassell, D. O'Hagan, C. R. Alving, and G. M. Glenn. 2000. Transcutaneous immunization with bacterial ADP-ribosylating exotoxins, subunits, and unrelated adjuvants. *Infect. Immun.* **68**: 5306–5313.
29. Sesardic, D., R. Winsnes, P. Rigsby, and R. Gaines-Das. 2001. Calibration of replacement International Standard and European Pharmacopoeia reference preparation for diphtheria toxoid, adsorbed. *Biologicals* **29**:107–122.
30. Sesardic, D., C. Prior, A. Daas, K. H. Buchheit, and National Institute for Biological Standards and Control, Hertfordshire, UK European Directorate for the Quality of Medicines, Council of Europe, Strasbourg, France. 2003. Collaborative study for establishment of the European Pharmacopoeia BRP batch 1 for diphtheria toxin. *PharmaEuropa Bio*. **1**:7–21.
31. Sesardic, D., S. Rijpkema, and B. P. Patel. 2007. New adjuvants: EU regulatory developments. *Expert Rev. Vaccines* **6**:849–861.
32. Spangler, B. D. 1992. Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol. Rev.* **56**:622–647.
33. Tierney, R., A. S. Beignon, R. Rappuoli, S. Muller, D. Sesardic, and C. D. Partidos. 2003. Transcutaneous immunization with tetanus toxoid and mutants of *Escherichia coli* heat-labile enterotoxin as adjuvants elicits strong protective antibody responses. *J. Infect. Dis.* **188**:753–758.
34. Winsnes, R., D. Sesardic, A. Daas, and P. Rigsby. 2002. A Vero cell method for potency testing of diphtheria vaccines. *Dev. Biol. (Basel)* **111**:141–148.