Transcutaneous Immunization with Cross-Reacting Material CRM$_{197}$ of Diphtheria Toxin Boosts Functional Antibody Levels in Mice Primed Parenterally with Adsorbed Diphtheria Toxoid Vaccine$^\dagger$

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$$^\dagger$

Division of Bacteriology, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Hertfordshire EN6 3QG, United Kingdom

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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 (CRM$_{197}$) 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 CRM$_{197}$ 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 CRM$_{197}$ 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 CRM$_{197}$ protein. These findings highlight the promising prospect of using booster administrations of CRM$_{197}$ 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, antitoxin 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 antitoxin 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 (CRM$_{197}$) 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). CRM$_{197}$ is licensed for human use as a carrier protein for several capsular polysaccharide antigens.
and is a promising vaccine candidate and potential alternative to conventional DTaX 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 nanoparticles (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 DTaX 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 DTaX vaccine given by the subcutaneous (s.c.) route.

**MATERIALS AND METHODS**

**Immunization procedures.** For parental priming, we used the WHO Third International Standard for DTaX (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 DTaX vaccine (2.5 IU/dose). Twelve weeks after priming, groups of mice were boosted s.c. with adsorbed DTaX 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 tissue followed by washing with tepid water.

**ELISA for measurement of antibody responses.** To measure the total anti-CRM197 and anti-DTaX 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 DTaX (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 above, and antigen-specific IgG antibodies were detected using a horseradish peroxidase-conjugated goat-mouse IgG antibody (catalog no. A-9044; Sigma) diluted 1:2000 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 (A405) 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 DTaX-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 DTaX as described above. Reference anti-mouse IgG1 and anti-IgG2a antibodies (Sigma, Dorset, United Kingdom) were (Sigma, Dorset, United Kingdom) were added to the plates. 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-DT antibody neutralizing activities.** The neutralizing capacity of anti-DT antibodies was measured using the Vero cell toxin neutralization assay as described in detail elsewhere (10, 30, 34). The neutralizing titer, defined as the dilution of samples, expressed as the reciprocal of the number of hemadsorbing 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 µg/ml 2-mercaptoethanol (Sigma). Viable splenocytes (2 × 10⁶ 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 DTaX (6,000 flocculation units/ml) for the group boosted s.c. with adsorbed DTaX 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 [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

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 (∼1/20th 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 parenteral 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).
with CRM\textsubscript{197} alone (Fig. 2). However, transcutaneous boost using CRM\textsubscript{197} with CT as the adjuvant did not significantly increase the anti-DTx response compared to the response observed with CRM\textsubscript{197} 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 CRM\textsubscript{197} 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 CRM\textsubscript{197} alone are comparable to those induced using CRM\textsubscript{197} with adjuvant or parenteral boost with adsorbed DTx\textsubscript{d} 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 DTx\textsubscript{d} 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 (CRM\textsubscript{197} alone) and the group given adsorbed DTx\textsubscript{d} vaccine. At week 26 (14 weeks postboost), neutralizing antibody levels had fallen by 63\% in the CRM\textsubscript{197}-plus-CT-treated group ($P < 0.005$), 62\% in the CRM\textsubscript{197}-plus-LTR72-treated group ($P < 0.05$), and 65\% in the alum-adsorbed DTx\textsubscript{d} 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 CRM\textsubscript{197} 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 CRM\textsubscript{197} alone and any of the groups treated with adjuvant (CT, LTR72, or adsorbed DTx\textsubscript{d}). At this time, there were no significant differences between adjuvant or immunization route in terms of inducing a neutralizing antidiiptheria response (Fig. 2).

TCI adjuvant influences the IgG subclass response to immunization with CRM\textsubscript{197}. 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 DTx\textsubscript{d} vaccine also induced antibodies that were predominantly of the IgG1 subclass. However, mice boosted using CRM\textsubscript{197} with CT as the adjuvant showed a significant reduction in the IgG1/IgG2a ratio compared to mice immunized with CRM\textsubscript{197} alone or with LTR72 as the adjuvant and compared to mice immunized parenterally with two doses of adsorbed DTx\textsubscript{d} vaccine, indicating a shift toward a Th1-type immune response in the presence of this adjuvant (Fig. 3C).

TCI with CRM\textsubscript{197} induces potent antigen-specific T-cell responses. Antigen-specific T-cell responses following booster immunization with CRM\textsubscript{197} or adsorbed DTx\textsubscript{d} 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 CRM\textsubscript{197} (with or without adjuvant) or subcutaneously with adsorbed DTx\textsubscript{d} 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 CRM\textsubscript{197} with or without adjuvant. However, at the highest dose of restimulating antigen (10 $\mu$g/ ml), proliferation induced by immunization with the adsorbed DTx\textsubscript{d} vaccine was significantly higher than that induced by transcutaneous boost with CRM\textsubscript{197} 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-$\gamma$) were detected in the supernatants of spleen cells isolated from mice boosted transcutaneously with CRM\textsubscript{197} 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
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
Values that were significantly different from the value of the group in the presence of 0.1, 1, or 10 µg/ml CRM197. Spleen cells 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.
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 afterboosting, 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.
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