INFECTION AND IMMUNITY, Mar. 1999, p. 1100–1106 Vol. 67, No. 3
0019-9567/99/$04.00+0

Transcutaneous Immunization with Bacterial ADP-Ribosylating Exotoxins as Antigens and Adjuvants

GREGORY M. GLENN,[1,2]* TANYA SCHARTON-KERSTEN,[1,2] RUSSELL VASSELL,[1,2] GARY R. MATYAS,[1] AND CARL R. ALVING[1]

Department of Membrane Biochemistry, Walter Reed Army Institute of Research, Washington, D.C. 20307-5100,[1] and IOMAI Corp., Washington, D.C. 20037[2]

Received 1 September 1998/Returned for modification 14 October 1998/Accepted 30 November 1998

Transcutaneous immunization (TCI) is a new technique that uses the application of vaccine antigens in a solution on the skin to induce potent antibody responses without systemic or local toxicity. We have previously shown that cholera toxin (CT), a potent adjuvant for oral and nasal immunization, can induce both serum and mucosal immunoglobulin G (IgG) and IgA and protect against toxin-mediated mucosal disease when administered by the transcutaneous route. Additionally, CT acts as an adjuvant for coadministered antigens such as tetanus and diphtheria toxoids when applied to the skin. CT, a member of the bacterial ADP-ribosylating exotoxin (bARE) family, is most potent as an adjuvant when the A-B subunits are present and functional. We now show that TCI induces secondary antibody responses to coadministered antigens as well as to CT in response to boosting immunizations. IgG antibodies to coadministered antigens were also found in the stools and lung washes of immunized mice, suggesting that TCI may target mucosal pathogens. Mice immunized by the transcutaneous route with tetanus fragment C and CT developed anti-tetanus toxoid antibodies and were protected against systemic tetanus toxin challenge. We also show that bAREs, similarly organized as A-B subunits, as well as the B subunit of CT alone, induced antibody responses to themselves when given via TCI. Thus, TCI appears to induce potent, protective immune responses to both systemic and mucosal challenge and offers significant potential practical advantages for vaccine delivery.

Transcutaneous immunization (TCI), introduction of antigens by topical application to intact skin, has many practical merits compared to injectable routes of administration. This needle-free method of vaccine delivery could decrease the risk of needle-borne diseases, reduce the complications related to physical skin penetration, and improve access to vaccination by eliminating the need for trained personnel and sterile equipment. As an initial step toward the development of this new route of immunization, we recently reported that cholera toxin (CT) acts as an adjuvant for coadministered antigens when applied to the surface of the skin (14).

CT is an 86-kDa heterodimeric protein which is secreted by the bacterium *Vibrio cholerae* when colonizing the small intestine, where the toxin induces massive fluid secretion by the intestinal epithelium (9, 23). CT is organized as an $A\text{-}B_5$ proenzyme with the ADP-ribosyltransferase activity contained in the A subunit and its target cell binding region located on the B subunit which binds to the ubiquitous cell membrane ganglioside G_{M1} (18, 22). While a profound rise in the level of intracellular cyclic AMP upon binding of CT to the ganglioside G_{M1} on the intestinal epithelia is thought to lead to fluid loss and diarrhea, the mechanism of its adjuvant effect in the immune system is not fully understood (25). CT is a member of the bacterial ADP-ribosylating exotoxin (bARE) family, which also includes *Escherichia coli* heat-labile enterotoxin (LT), *Bordetella pertussis*-derived pertussis toxin (PT), *Pseudomonas aeruginosa* exotoxin A (ETA), and *Corynebacterium diphtheria*-derived diphtheria toxin (22).

When administered perorally or intranasally, CT induces antibody responses against both itself and coadministered proteins and is thus considered a potent mucosal adjuvant (11, 31). The perceived toxicity of CT and the related toxin LT has limited the widespread use of these proteins as vaccine components and adjuvants and has led to mucosal strategies involving nontoxic mutants (10, 13, 24, 31) and purified B subunits (19, 32). However, we have recently found that application of CT to the skin induces potent immune responses without evidence of the systemic toxicities that accompany its use via oral, nasal, or parenteral routes (14). Thus, TCI allows the use of native CT as an adjuvant without causing the expected side effects.

We have shown in previous studies that application of CT along with other proteins induces antibody responses against both the toxin and coadministered proteins (14) and that anti-CT antibodies are sufficient to protect the immunized animals from a lethal mucosal challenge with the toxin (15). We now show that the use of CT as an adjuvant results in classic secondary antibody responses to boosting, the presence of mucosal antibodies to coadministered antigens, and systemic protection.

MATERIALS AND METHODS

Animal care and use. The work described in this paper was conducted under a protocol approved by the Institute's Laboratory Animal Care and Use Committee in accordance with the *Guide for the Care and Use of Laboratory Animals* (28a) in facilities that are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. The animals were cared for by the Department of Animal Medicine, Walter Reed Army Institute of Research, with biosafety level 2 precautions.

Immunization and antigens. CT, CT B subunit (CTB), CTA, ETA, diphtheria toxoid (DT), tetanus fragment C (tetC), tetanus toxoid, and tetanus toxin were obtained from LIST Biologicals (Campbell, Calif.), and bovine serum albumin (BSA) and LT were obtained from Sigma (St. Louis, Mo.). BALB/c mice, 6 to 8 weeks of age, were shaved on the dorsum with a no. 40 clipper and rested for 48 h. The mice were anesthetized with ketamine-xylazine during the immunization procedure to prevent grooming. The skin was wetted with 100 μl of immunizing solution placed on the shaved skin over a 2-cm^2 area and left for 2 h. The mice were then extensively washed with approximately 1 liter of lukewarm tap water, patted dry, and washed again. No adverse effects from the shaving, anesthesia,

* Corresponding author. Mailing address: Department of Membrane Biochemistry, Walter Reed Army Institute of Research, Bldg. 40, Rm. 3049, 14th and Dahlia Sts. NW, Washington, DC 20307-5100. Phone: (202) 782-3137. Fax: (202) 782-1890. E-mail: gglenn@iomai.com.

TABLE 1. Antibody responses to CT, CT subunits, and other bAREs[a]

Immunizing antigen	Antibody specificity	Mean IgG titer in serum (ELISA units)	SEM
CT	Anti-CT	39,828	17,826–44,838
CTB	Anti-CTB	7,480	3,756–14,896
rCTB[b]	Anti-CTB	9,324	5,271–13,372
CTA	Anti-CTA	0	0
LT	Anti-LT	22,461	20,262–27,167
ETA	Anti-ETA	3,758	1,951–77,240
BSA	Anti-BSA	0	0

[a] BALB/c mice ($n = 5$) were immunized with 100 μg of antigen at 0 and 3 weeks. Antibody titers were measured by ELISA at 6 weeks. The results are reported as the geometric mean ± SEM of individually assayed sera in ELISA.
[b] rCTB, recombinant CTB.

immunization, or washing procedures were observed. Neither erythema nor induration was seen at the immunization site for up to 72 h after the antigen exposure.

ELISA. Antibody levels against CT, CTB, CTA, LT, ETA, BSA, DT, and tetC were determined by an enzyme-linked immunosorbent assay (ELISA). Immulon-2 polystyrene plates (Dynatech Laboratories, Chantilly, Va.) were coated with 0.1 μg of antigen in saline per well, incubated at room temperature overnight, blocked with 0.5% casein–Tween 20, and washed; serial dilutions of sera were applied; and the plates were incubated for 2 h at room temperature. Specific immunoglobulin G (IgG) heavy-plus-light-chain (H+L) antibody was detected by using horseradish peroxidase-linked goat anti-mouse IgG (H+L) (Bio-Rad, Richmond, Calif.) and revealed after 30 min with 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) substrate (ABTS; Kirkegaard & Perry, Gaithersburg, Md.), and the reaction was stopped with 1% sodium dodecyl sulfate. The plates were read at 405 nm. IgA antibody levels were determined as above with goat anti-mouse IgA (Zymed, South San Francisco, Calif.) as the secondary antibody. Results reported in ELISA units were determined by using the inverse dilution of the serum that yields an optical density of 1.0. In all cases, ELISAs were conducted to discount the role of cross-reactivity between coadministered antigens (data not shown).

Lung washes and stool collection. Lung washes were obtained from immunized mice. The mice were sacrificed, the trachea was transected, 22-gauge polypropylene tubing was inserted, and phosphate-buffered saline was infused to gently inflate the lungs. The infused material was then withdrawn and reinfused for a total of three cycles and stored at −20°C. Stool pellets were collected the day before challenge after spontaneous defecation, weighted, homogenized in 1 ml of phosphate-buffered saline per 100 mg of fecal material, and centrifuged, and the supernatant was collected and stored at −20°C.

Tetanus toxin challenge. Mice were challenged with tetanus toxin as previously described (12). The mice were injected subcutaneously in the scruff of the neck with 10 ng of tetanus toxin in nutrient broth-borate buffer (1:1) and were observed for 7 days. Those that developed severe paralysis were euthanized.

^{125}I labeling of CT. CT was labeled with chloramine-T as previously described (8). The resulting activity of the ^{125}I-labeled CT was shown to be 1.5×10^6 counts/min/mg.

Statistical analysis. Unless otherwise indicated, the data shown are the geometric means and standard errors of the mean (SEM) from values obtained with individual animals. Antibody titers in groups were compared by an unpaired two-tailed Student t test, and $P < 0.05$ was regarded as significant.

RESULTS

Antibody response to CT and other ADP-ribosylating exotoxins. We recently reported that CT placed on the skin in saline solution acts as a potent immunogen and elicits a strong systemic anti-CT antibody response (14) (Table 1). By contrast, BSA applied in the same fashion does not induce a readily detectable anti-BSA response. Since CT is a member of the bARE family, we tested whether other proteins in this group might also induce circulating antibodies after application to the skin. Similar to CT, purified CTB induced a strong antibody response (Table 1). Although purified preparations of CTB may contain small amounts (<0.01%) of enzymatic CTA, recombinant CTB, which lacks CTA entirely, was equally immunogenic compared to purified CTB. In contrast, purified CTA induced no anti-CTA IgG titers, suggesting that CTB activity is important for TCI. LT, which is similar to CT in size, amino acid sequence, and receptor binding (4, 31), induced a potent antitoxin antibody response in the sera. ETA, which contains both A and B domains on the same peptide and bears little structural homology to LT or CT (22), also induced a transcutaneous antibody response against itself. Therefore, as a group, these members and subunits of the bARE family appear active when applied as antigens for TCI.

Antibody dose response to CT and LT. Initial studies with CT as an immunogen indicated that 100 μg of antigen was sufficient to induce high levels of antibodies in serum (14, 15). To describe the dose-response relationship between CT and its resulting humoral response, different amounts of CT (1 to 300 μg) in a fixed volume of saline were applied to the skin. The mice were immunized and boosted 4 and 8 weeks later. No significant difference was found in the responses to 300 μg and to 100 μg, but the antibody titer at all other doses was significantly different (Fig. 1). A clearly detectable response was observed in doses as low as 3 μg. Similarly, LT induced antigen-

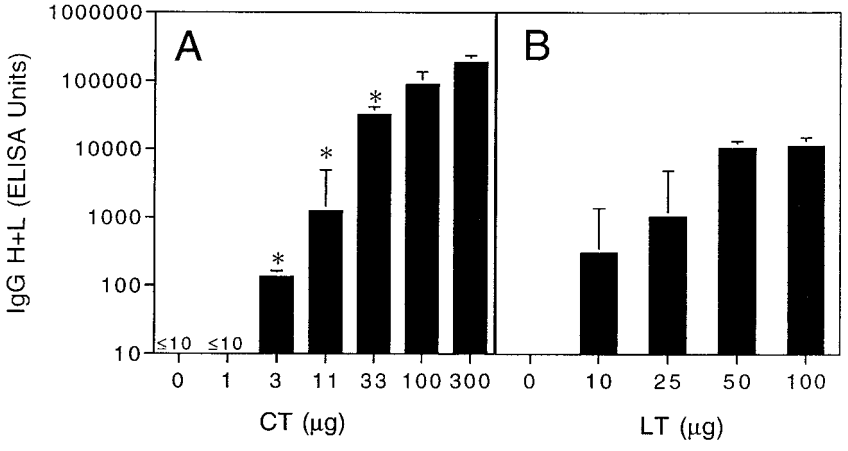


FIG. 1. Dose-dependent antibody response to CT (A) or LT (B) in mice immunized by the transcutaneous route. (A) The mice ($n = 5$) were immunized at 0, 4, and 8 weeks, and serum was analyzed for anti-CT IgG (H+L) titers at 12 weeks. (B) The mice ($n = 5$) were immunized at 0 weeks, and serum was analyzed for anti-LT IgG (H+L) titers 3 weeks later. The results shown are the geometric mean and SEM of IgG titers measured in sera from five individual animals and reported in ELISA units, the inverse dilution at which the absorbency is equal to 1.0 at 405 nm. An asterisk denotes a statistically significant difference ($P \leq 0.05$) in antibody titer compared to the response in the high-dose groups.

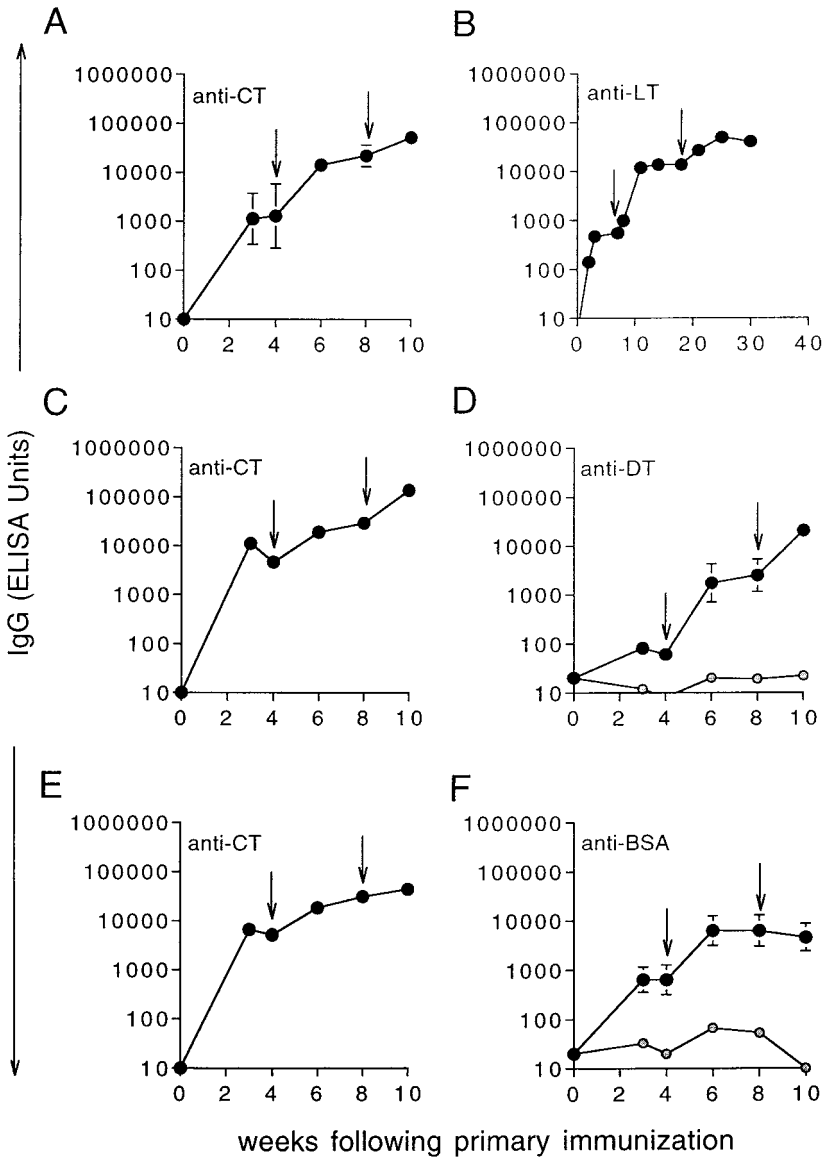


FIG. 2. Kinetics of the IgG (H+L) antibody response to CT (A, C, E), LT (B), DT (D), or BSA (F) in animals immunized and boosted (arrows) by the transcutaneous route. The mice ($n = 5$) were immunized with CT alone (100 μg) (A), LT alone (100 μg) (B), CT plus DT (100 μg of CT + 100 μg of DT [black circles]) (C and D), DT alone (100 μg [gray circles]) (D), CT plus BSA (100 μg of CT + 200 μg of BSA [black circles]) (E and F), or BSA alone (200 μg [gray circles]) (F). Gray circles in panels D and F indicate antibody levels to DT or BSA, respectively, in mice vaccinated without using CT as the adjuvant. Antibody titers were measured by ELISA at multiple time points. The results are reported as the mean ± SEM. Similar results were obtained in two independent experiments.

specific antibody titers in a dose-dependent manner after a single immunization, and 10 μg was clearly immunogenic.

Kinetics of the antibody response to CT and coadministered antigen. Previous studies indicated that CT acts as an adjuvant when coadministered with another protein such as DT (14). In the present study, we examined the kinetics of the antibody response associated with TCI. The potent antibody response to a priming dose with CT as an immunogen was further increased after the second and third immunizations (Fig. 2A). By comparison, the antibody response to LT as an immunogen was less pronounced but also increased after the second and third immunizations. The secondary antibody response to CT, when administered as an adjuvant with DT or BSA, was not diminished compared to the response to CT alone (Fig. 2C and E). The antibody response to the coadministered antigens DT and BSA was clearly enhanced by using CT as an adjuvant and showed a secondary antibody response upon boosting (Fig. 2D and F). The antibody response to DT and BSA could be boosted with CT as an adjuvant despite the high levels of preexisting CT antibodies (Fig. 2D and F).

Reimmunization with coadministered antigens. The ability to reimmunize previously immunized animals with another antigen and CT as an adjuvant was evaluated with DT and BSA. Mice were immunized and boosted on the skin with DT, with CT as an adjuvant. The same mice were then reimmunized on the skin with BSA, also with CT as an adjuvant. As shown in Fig. 3, the mice initially produced both anti-CT and anti-DT antibodies as expected. After the same mice were immunized and boosted with BSA plus CT adjuvant, they produced anti-BSA antibodies of a magnitude similar to naive mice vaccinated only with CT and BSA, as shown in the previous experiment (Fig. 2F). Thus, reimmunization with a sec-

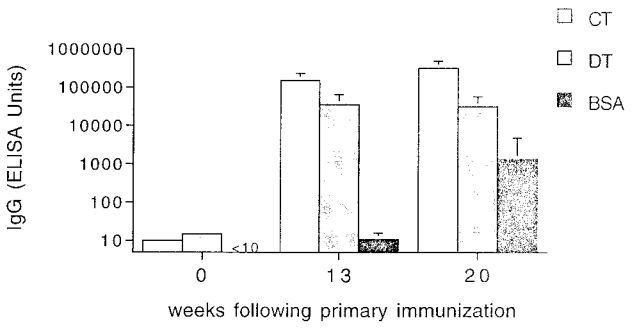


FIG. 3. TCI of mice with preexisting anti-CT and anti-DT titers with a third, unrelated antigen (BSA). The mice were immunized by the transcutaneous route at 0, 4, and 8 weeks with CT (100 μg) and DT (100 μg) and then exposed to CT (100 μg) and BSA (200 μg) at 13 and 17 weeks after the first immunization. Serum collected from the animals at 0, 13, and 20 weeks was analyzed. The results are reported as the mean ± SEM for groups of five mice. Similar results were obtained with BSA as the primary immunogen and DT as the second immunogen.

ond coadministered antigen with CT as an adjuvant appears feasible in the context of TCI.

Antibody response to CT and coadministered antigens at the mucosa. We recently reported that CT administered via TCI induces protective anti-CT antibodies detectable at the mucosal surfaces (15). To determine whether CT also induces an antibody response against coadministered antigen detectable at the mucosal surfaces, mice were immunized transcutaneously with CT as an adjuvant for DT and the mucosal (lung and stool) anti-CT and anti-DT IgG and IgA titers were evaluated. Anti-CT IgG and IgA were detected in the lung wash and stool samples from mice immunized with CT alone or with CT as an adjuvant for DT (Fig. 4A and B). Animals exposed to both CT and DT exhibited anti-DT IgG responses in the lung wash and stool samples (Fig. 4C). Anti-DT IgA was not found in these mice, and IgG and IgA were not detected in the stool or lung wash samples from animals exposed to DT alone.

Tetanus toxin Challenge. Previous studies have shown that TCI can protect against lethal mucosal toxin challenge (15). In this experiment, we tested the ability of TCI to confer protection against systemic tetanus toxin challenge. A papain derivative of tetanus toxin, tetC, which has been shown to be a protective antigen (12), was used with CT as an adjuvant. As shown in Table 2, all mice immunized with tetC plus CT produced anti-tetC and anti-tetanus toxoid antibodies. Two mice immunized with tetC alone produced a low-level anti-tetC response. In accordance with these results, all mice immunized with CT plus tetC were protected against tetanus toxin challenge, as were the two responders in the tetC alone group. No protection was seen in mice immunized with CT or CT plus sequestrin, an irrelevant protein (data not shown).

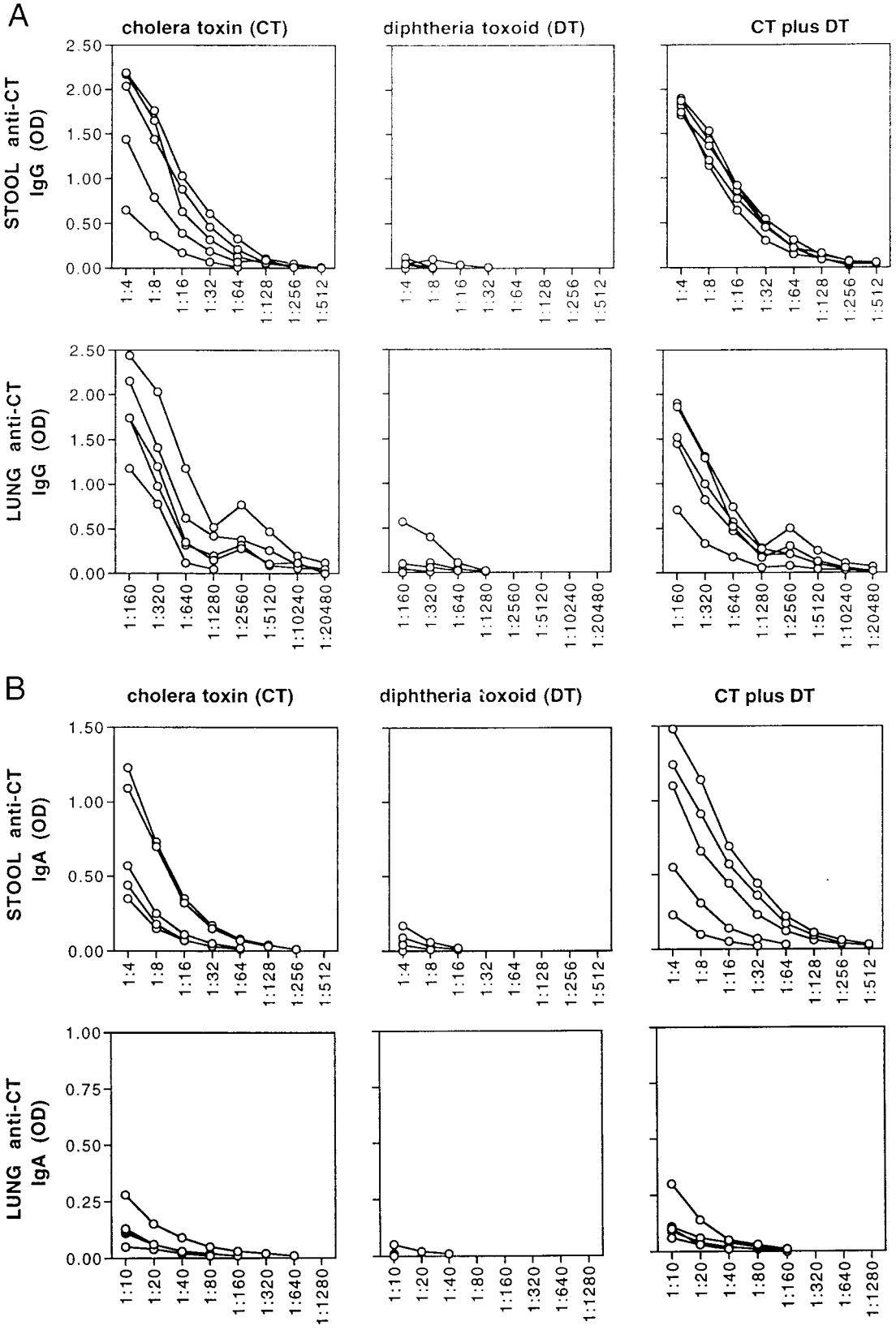
Fate of ^{125}I-labeled CT administered by TCI. The possibility that oral immunization occurred through grooming was evaluated by using ^{125}I-labeled CT to track its fate. CT is exquisitely sensitive to inactivation by gastric acid, and for this reason oral immunization with CT is normally performed with bicarbonate to neutralize the pH in the stomach (5). Therefore, it seemed unlikely that ingestion of the CT remaining after extensive washing would account for the potent systemic antibody response observed following TCI. Previous studies performed in our laboratory revealed that mice fed 10 μg of CT in saline developed a detectable but significantly lower antibody response to CT than did animals given CT via TCI (<1,000 and 39,000 anti-CT ELISA units, respectively). In the present study, mice were anesthetized and immunized on the back with 100 μg of ^{125}I-labeled CT containing 1.5×10^6 counts/min/mg. Control mice remained anesthetized for 6 h to exclude grooming, and experimental mice were anesthetized for 1 h and then allowed to groom after being washed. The mice were sacrificed at 6 h, and their organs were weighed and assayed for ^{125}I on a Packard gamma counter. A range of 2 to 3 μg of CT remained on the skin at the site of immunization after washing (14,600 cpm/mg of tissue), while a maximum of 0.5 μg of CT was detected in the stomach (661 cpm/mg of tissue) and intestines (9 cpm/mg of tissue). Additionally, guinea pigs, animals that are unable to groom their backs, developed high levels of anti-CT antibodies in response to TCI (unpublished observations). These data suggest that oral immunization does not significantly contribute to the circulating antibodies detected when CT is placed on the skin.

DISCUSSION

In previous studies, we found that TCI results in potent antibody responses (14) and that protection against CT-mediated mucosal disease can be elicited (15). In the present study, we show that TCI induces secondary antibody responses to boosting (Fig. 2), responses to coadministered antigens that can be detected at the mucosa (Fig. 3), and protection against systemic toxin challenge (Table 2). These immunological responses to TCI are desirable outcomes of vaccine adjuvant use, and thus TCI may have broad application to vaccine strategies requiring antibody responses. Although the underlying immune mechanisms leading to TCI remain to be determined, the role of Langerhans cells acting as antigen-presenting cells activated by adjuvants may explain the phenomenon of immunization through intact skin, since these cells are believed to be the only antigen-presenting cells in uninflamed skin (33). Thus, TCI may provide powerful access to the immune system as well as opening a new route for vaccine delivery.

Most vaccines require multiple boosts to achieve protective levels of antibodies. We found that TCI results in secondary antibody responses to boosting and that high levels of antibodies to the coadministered antigens, DT or BSA, can be achieved (Fig. 2). Interference with the ability to boost coadministered antigens by the simultaneous boosting of CT antibodies was not seen despite the high level of response to CT. Similarly, reimmunization with BSA after the induction of anti-DT antibodies was demonstrated despite high preexisting levels of anti-CT antibodies. Thus, a potent multivalent antigen-specific response to TCI was achieved through boosting of the secondary antibody response without interference.

The tetanus challenge model was selected to evaluate whether TCI could produce a protective immune response against a vaccine-preventable disease. The correlate for systemic protection against tetanus is known to be the induction of antitoxin IgG in serum (26). Both antigen-specific IgG and protection were readily induced by TCI, suggesting that this method induces functional responses that may be used for other vaccines. Since TCI induced both systemic and mucosal antibodies to the coadministered antigens, the TCI approach may be beneficial for diseases stemming from mucosal pathogens. Protection against toxin-mediated diseases, such as diphtheria, tetanus, and possibly pertussis, is known to be mediated in large part by antitoxin antibodies (30). The ability of TCI to induce high levels of anti-CT antibodies has raised the possibility of an effective antitoxin enteric vaccine (15). Anti-CT immunity can be completely protective in animals (17, 29) and clearly contributes to immunity in resistant humans (6). Although the correlates for protection against other vaccine-preventable diseases such as diphtheria and *Haemophilus influenzae* b infection are also known to be antigen-specific IgG in serum (16,


A
cholera toxin (CT)
diphtheria toxoid (DT)
CT plus DT
STOOL anti-CT IgG (OD)
LUNG anti-CT IgG (OD)
B
cholera toxin (CT)
diphtheria toxoid (DT)
CT plus DT
STOOL anti-CT IgA (OD)
LUNG anti-CT IgA (OD)

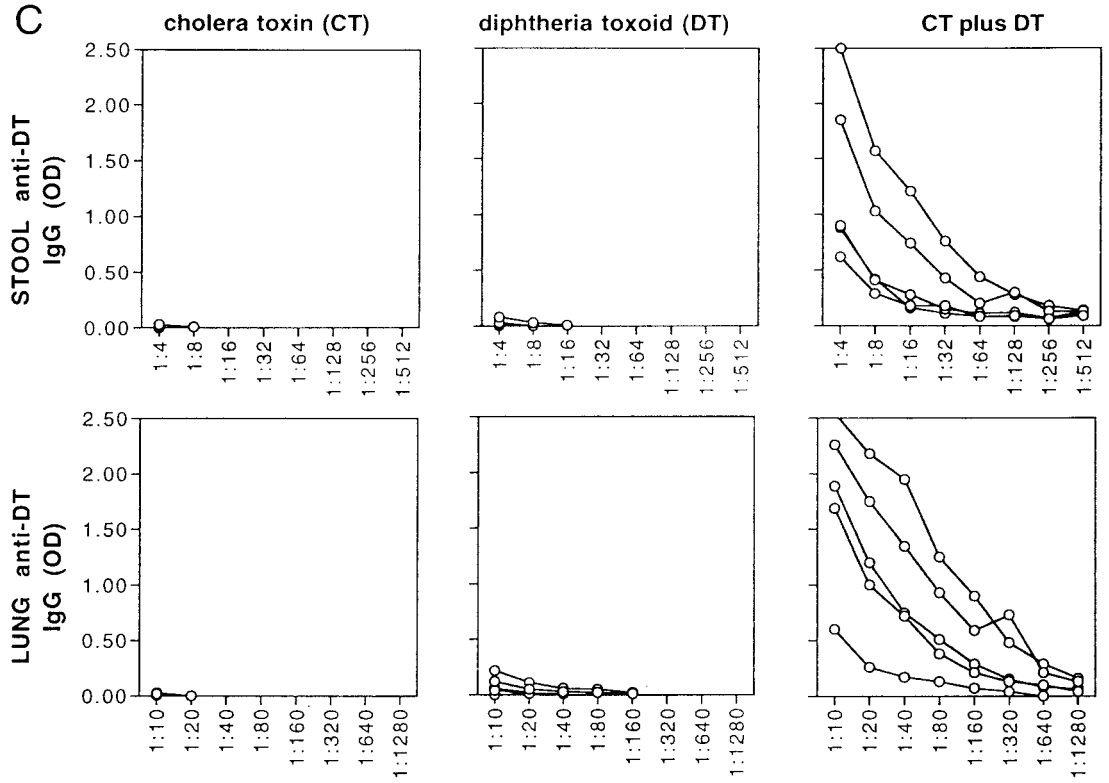


FIG. 4. Responses to CT and coadministered antigens DT at the mucosa. Mice ($n = 5$) were immunized with CT (100 μg) (left), DT (100 μg) (middle), or CT plus DT (100 μg each) (right) at 0, 8, and 18 weeks by TCI. At 30 weeks later, stool samples and lung wash extracts were collected and analyzed for anti-CT (A and B) and anti-DT (C) Ig. Antigen-specific IgG (A and C) and IgA (B) titers were measured. Curves showing the titers from individual animals are plotted. Control stool preimmune samples from mice and control lung washes from mice immunized with an irrelevant protein, ricin A subunit, had optical densities of 0. The results are representative of data observed in two independent experiments.

30), the presence of mucosal IgG is also likely to be important for protection against these mucosal pathogens (20).

The presence of antigen-specific antibodies in the mucosa after TCI may be due to local production by antibody-secreting cells (IgA) or through transudation of circulating immunoglobulin from the sera (IgG or IgA). In general, mucosal IgG is thought to be transudative (28), and such responses are clearly protective against influenza (7). On the other hand, IgA detected at the mucosal level is usually presumed to be of local origin (31), but this remains to be determined for the anti-CT IgA produced by TCI, since high levels of anti-IgA are present in the sera of immunized mice (15). Thus, although TCI is similar to intramuscular immunization in that TCI induces serum IgG to coadministered antigen, TCI raises the levels of serum and mucosal IgG antibodies to coadministered antigens as well as the levels of serum and mucosal IgA antibodies to CT.

A structurally dissimilar bARE, ETA, was used to determine if the phenomenon of TCI might be more generalized. ETA, derived from *P. aeroginosa*, is a single 613-amino-acid peptide with A and B domains on the same peptide. This is unlike CT and LT, which are composed of large (86-kDa), noncovalently linked subunits with A-B_5 stoichiometry (22). ETA binds to an entirely different receptor, the α_2-macroglobulin receptor/low-density lipoprotein receptor-related protein (21). Despite the dissimilarities in size, structure, and binding target between ETA and CT, ETA also induced a transcutaneous antibody response. Thus, TCI is not entirely dependent on CT or LT binding of ganglioside G_{M1}, as suggested by the poor response to CTA alone, and the adjuvant effects seen with TCI may not be exclusively dependent on CT. bAREs such as ETA, LT, and other adjuvants may provide alternative antigens and adjuvants on the skin and may lead to new strategies for induction of protective immunity. Although the optimal dose of CT as an adjuvant is not known, CT is clearly active as an antigen at doses as low as 3 μg (Fig. 1), indicating that lower doses of CT or other adjuvants may be effective.

Several experiments were designed to evaluate the potential

TABLE 2. Protection against subcutaneous challenge with tetanus toxin[a]

Mouse tag no.	Immunization antigen	Antibody titer (ELISA units)			Survival at 10 days
		Anti-CT	Anti-tetC	Anti-TT	
5401	tetC	26	48	36	−
5402	tetC	35	677	422	+
5403	tetC	19	15	18	−
5404	tetC	21	43	30	−
5405	tetC	17	110	70	+
5406	CT + tetC	277,606	7,469	1,402	+
5407	CT + tetC	147,439	5,499	5,213	+
5408	CT + tetC	34,134	>12,800	>12,800	+
5409	CT + tetC	1,488,896	>12,800	>12,800	+
5410	CT + tetC	36,079	2,359	84	+

[a] Mice were immunized with TetCT (100 μg) and/or CT (100 μg) at 0, 4, 9, and 15 weeks. Three weeks later (18 weeks), the animals were bled for analysis of anti-CT, TetC, and tetanus toxoid Ig (H+L). The mice were then challenged with tetanus toxin (10 ng/animal) by the subcutaneous route at the nape of the neck and evaluated for survival.

role of grooming in the antibody response in mice immunized via TCI. The experiments have shown that mice immunized by oral feeding or gastric infusion in the absence of buffering of the acidic gastric environment develop far lower antibody titers to CT than do those given CT by TCI. These low antibody responses suggest that CT, as was found in other studies, does not tolerate the low gastric pH (5) and that oral intake of the holotoxin does not play an important role in the antibodies induced by TCI. The ^{125}I labeling of CT showed that the actual amount of CT which might be ingested is at least 1 log unit below the dose delivered in oral feeding experiments. The maximum amount of ingested ^{125}I-labeled CT was calculated to be 0.5 μg, far below the dose required to induce a high anti-CT antibody response when administered orally without bicarbonate. Because mice could be immunized through nicks in the skin, the animals were shaved 48 h before immunization, and the rare nicked mouse was eliminated from the experiment. More importantly, several studies have also shown that mice can be effectively be immunized on the unshaved surface of the ear (15).

TCI appears to offer a new method for the delivery of vaccines, with special advantages for multiple boosting immunizations or multivalent-vaccine delivery. TCI may avoid the need for sterile techniques and equipment and highly trained personnel, thereby circumventing the problems of blood-borne spread of disease from the reuse of both needles and jet injectors (1–3, 35) and decreasing the barriers to immunization. Intradermal injection and jet injector systems, which can achieve immune responses superior to that produced by the intramuscular route (27, 34), presumably target anatomical sites which are similar to those targeted by TCI, suggesting that TCI may be an immunologically sound approach to vaccination. Thus, TCI appears to induce potent, functional immune responses coupled with significant practical advantages for vaccine delivery.

ACKNOWLEDGMENTS

We thank Deborah Walwender and Elaine Morrison for technical assistance.

The work presented was supported and performed under a Cooperative Research and Development Agreement between Walter Reed Army Institute of Research and Medical Technologies Practice Patterns Institute, Washington, D.C.

REFERENCES

1. **Aylward, B., J. Lloyd, M. Zaffran, R. McNair-Scott, and P. Evans.** 1995. Reducing the risk of unsafe injections in immunization programmes: financial and operational implications of various injection technologies. Bull. W.H.O. **73:**531–540.
2. **Brink, P. R. G., A. M. van Loon, J. C. M. Trommelen, F. W. J. Gribnau, and I. R. O. Smale-Novakova.** 1985. Virus transmission by subcutaneous jet injection. J. Med. Microbiol. **20:**393–397.
3. **Centers for Disease Control.** 1986. Hepatitis B associated with jet gun injection—California. Morbid. Mortal. Weekly Rep. **35:**373–376.
4. **Clements, J. D., R. J. Yancey, and R. A. Finkelstein.** 1980. Properties of homogeneous heat-labile enterotoxin from *Escherichia coli*. Infect. Immun. **29:**91–97.
5. **Clements, J. D., M. Jertborn, D. Sack, B. Stanton, J. Holmgren, M. R. Khan, and S. Huda.** 1986. Effect of neutralization of gastric acid on immune responses to an oral B subunit, killed whole cell cholera vaccine. J. Infect. Dis. **154:**175–178.
6. **Clements, J. D., D. A. Sack, J. R. Harris, J. Chakraborty, P. K. Neogy, B. Stanton, N. Huda, M. U. Khan, A. K. Bradford, M. Ansaruzzaman, M. Yunus, M. R. Rao, A.-M. Svennerholm, and J. Holmgren.** 1988. Cross-protection by B subunit-whole cell cholera vaccine against diarrhea associated with heat-labile toxin-producing enterotoxigenic *Escherichia coli*: results of a large-scale field trial. J. Infect. Dis. **158:**372–377.
7. **Clements, M. L.** 1992. Influenza vaccines, p. 129–150. *In* R. W. Ellis (ed.), Vaccines: new approaches to immunological problems. Butterworth-Heinemann, Boston, Mass.
8. **Cuatrecasas, P.** 1973. Interaction of *Vibrio cholerae* enterotoxin with cell membranes. Biochemistry **12:**3547–3558.
9. **De, S. N.** 1959. Enterotoxicity of bacteria-free culture-filtrate of *Vibrio cholerae*. Nature **183:**1533–1534.
10. **Dickinson, B. L., and J. D. Clements.** 1995. Dissociation of *Escherichia coli* heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. Infect. Immun. **63:**1617–1623.
11. **Elson, C. O., and W. Ealding.** 1984. Cholera toxin feeding did not induce oral tolerance in mice and abrogated oral tolerance to an unrelated protein antigen. J. Immunol. **133:**2892–2897.
12. **Fairweather, N. F., V. A. Lyness, and D. J. Maskell.** 1987. Immunization of mice against tetanus with fragments of tetanus toxin synthesized in *Escherichia coli*. Infect. Immun. **55:**2541–2545.
13. **Giuliani, M. M., G. Del Giudice, V. Giannelli, 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.
14. **Glenn, G. M., M. Rao, G. R. Matyas, and C. R. Alving.** 1998. Skin immunization made possible by cholera toxin. Nature **391:**851.
15. **Glenn, G. M., T. Scharton-Kersten, R. Vassell, C. P. Mallet, T. L. Hale, and C. R. Alving.** 1998. Transcutaneous immunization with cholera toxin protects mice against lethal mucosal toxin challenge. J. Immunol. **161:**3211–3214.
16. **Goldblatt, D., A. R. Vaz, and E. Miller.** 1998. Antibody avidity as a surrogate maker of successful priming by *Haemophilus influenzae* type b conjugate vaccines following infant immunization. J. Infect. Dis. **177:**1112–1115.
17. **Holmgren, J., A.-M. Svennerholm, O. Ouchterlony, A. Andersson, G. Walletstrom, and U. Westerberg-Berndtsson.** 1975. Antitoxic immunity in experimental cholera: protection and serum and local antibody responses in rabbits after enteric and parenteral immunization. Infect. Immun. **12:**1331–1340.
18. **Holmgren, J., and A.-M. Svennerholm.** 1977. Mechanisms of disease and immunity in cholera: a review. J. Infect. Dis. **136:**S105–S112.
19. **Holmgren, J., A.-M. Svennerholm, M. Jertborn, J. Clements, D. A. Sack, R. Salenstedt, and H. Wigzell.** 1992. An oral B subunit: whole cell vaccine against cholera. Vaccine **10:**911–914.
20. **Kauppi, M., J. Eskola, and H. Kayhty.** 1995. Anticapsular polysaccharide antibody concentrations in saliva after immunization with *Haemophilus influenzae* type b conjugate vaccines. Pediatr. Infect. Dis. J. **14:**286–294.
21. **Kounnas, M. Z., R. E. Morris, M. R. Thompson, D. J. FitzGerald, D. K. Strickland, and C. B. Saelinger.** 1992. The α_2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes *Pseudomonas* exotoxin A. J. Biol. Chem. **267:**12420–12423.
22. **Krueger, K. M., and J. T. Barbieri.** 1995. The family of bacterial ADP-ribosylating exotoxins. Clin. Microbiol. Rev. **8:**34–47.
23. **Levine, M. M., J. B. Kaper, R. E. Black, and M. L. Clements.** 1983. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. Microbiol. Rev. **47:**510–550.
24. **Lobet, Y., C. W. Cluff, and W. Cieplak.** 1991. Effect of site-directed mutagenic alterations on ADP-ribosyltransferase activity of the A subunit of *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **59:**2870–2879.
25. **Lycke, N.** 1997. The mechanism of cholera toxin adjuvanticity. Res. Immunol. **148:**504–520.
26. **McComb, J. A.** 1964. The prophylactic dose of homologous tetanus antitoxin. N. Engl. J. Med. **270:**175–178.
27. **Miller, K. D., R. D. Gibbs, M. M. Mulligan, T. B. Nutman, and D. P. Francis.** 1983. Intradermal hepatitis B virus vaccine: immunogenicity and side effects in adults. Lancet **ii:**1454–1456.
28. **Murphy, B. R., D. L. Nelson, P. F. Wright, E. L. Tierney, M. A. Phelan, and R. M. Chanock.** 1982. Secretory and systemic immunological response in children infected with live attenuated influenza A virus vaccines. Infect. Immun. **36:**1102–1108.

28a. **National Research Council.** 1996. Guide for the care and use of laboratory animals. National Research Council, Washington, D.C.

29. **Pierce, N. F., E. A. Kaniecki, and R. S. Northrup.** 1972. Protection against experimental cholera by antitoxin. J. Infect. Dis. **126:**606–616.
30. **Schneerson, R., J. B. Robbins, J. Taranger, T. Lagergard, and B. Trollfors.** 1996. A toxoid vaccine for pertussis as well as diphtheria? Lessons to be relearned. Lancet **348:**1289–1292.
31. **Snider, D. P.** 1995. The mucosal adjuvant activities of ADP-ribosylating bacterial enterotoxins. Crit. Rev. Immunol. **15:**317–348.
32. **Tamura, S., H. Funato, T. Nagamine, C. Aizawa, and T. Kurata.** 1989. Effectiveness of cholera toxin B subunit as an adjuvant for nasal influenza vaccination despite pre-existing immunity to CTB. Vaccine **7:**503–505.
33. **Udey, M. C.** 1997. Cadherins and Langerhans cell immunobiology. Clin. Exp. Immunol. **107**(Suppl. 1)**:**6–8.
34. **Whittle, H. C., W. H. Lamb, and R. W. Ryder.** 1987. Trials of intradermal hepatitis vaccines in Gambian children. Ann. Trop. Paediatr. **7:**6–9.
35. **Zachoval, R., F. Deinhardt, L. Gurtler, J. Eisenburg, and G. Korger.** 1988. Risk for virus transmission by jet injection. Lancet **ii:**189. (Letter.)

Editor: R. N. Moore