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Transcutaneous Immunization with *Clostridium difficile* Toxoid A Induces Systemic and Mucosal Immune Responses and Toxin A-Neutralizing Antibodies in Mice▽

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***Clostridium difficile* is the leading cause of nosocomial infectious diarrhea. *C. difficile* produces two toxins (A and B), and systemic and mucosal anti-toxin A antibodies prevent or limit *C. difficile*-associated diarrhea. To evaluate whether transcutaneous immunization with formalin-treated *C. difficile* toxin A (CDA) induces systemic and mucosal anti-CDA immune responses, we transcutaneously immunized three cohorts of mice with CDA with or without immunoadjuvantative cholera toxin (CT) on days 0, 14, 28, and 42. Mice transcutaneously immunized with CDA and CT developed prominent anti-CDA and anti-CT immunoglobulin G (IgG) and IgA responses in serum and anti-CDA and anti-CT IgA responses in stool. Sera from immunized mice were able to neutralize *C. difficile* toxin A activity in an in vitro cell culture assay. CDA itself demonstrated adjuvant activity and enhanced both serum and stool anti-CT IgA responses. Our results suggest that transcutaneous immunization with CDA toxoid may be a feasible immunization strategy against *C. difficile*, an important cause of morbidity and mortality against which current preventative strategies are failing.**

Clostridium difficile is a spore-forming, gram-positive, anaerobic bacillus and the leading cause of nosocomial diarrhea and colitis in the industrialized world. More than 300,000 cases of *C. difficile*-associated diarrhea are reported each year in the United States alone (3, 40, 57). Complications of *C. difficile*-associated diarrhea (CDAD) include pseudomembranous colitis, toxic megacolon, systemic inflammatory response syndrome, and death. Broad-spectrum antibiotic usage, hospitalization, advanced age, and comorbidities increase the risk of acquiring CDAD (32–34). Recently, a new, highly virulent strain of *C. difficile*, BI/NAP1/r027, has emerged and has been associated with outbreaks of severe nosocomial CDAD (4, 5, 36, 38, 55). No vaccine effective at preventing *C. difficile* disease is currently commercially available, and measures to prevent *C. difficile*-associated diarrhea through patient isolation and implementation of hand hygiene and contact precautions have had variable and often limited success (2, 12, 24). The ongoing increase in the annual reported incidence of nosocomial CDAD in the United States may in large part reflect this failure of current disease control measures (39).

C. difficile expresses two major virulence factors, toxin A and toxin B. These large toxins (toxin A, 308 kDa; toxin B, 270 kDa) function as glucosyltransferases that inactivate Rho, Rac, and Cdc42 within eukaryotic target cells, leading to actin polymerization, opening of tight junctions, and ultimately cell death (10, 54). Toxin A initiates intestinal epithelial damage and mucosal disruption that allows toxin B to gain access to underlying cells (37). A carboxyl-terminal 800-amino-acid portion of toxin A mediates binding of toxin A to receptors on epithelial cell surfaces (11, 30, 52). Monoclonal and polyclonal antibodies directed against this receptor-binding region of toxin A abrogate toxin activity and prevent clinical disease in animals (8, 13, 43). Antibodies against *C. difficile* are present in a majority of adults and older children, and serum immunoglobulin G (IgG) antibodies directed against toxin A are associated with protection against CDAD (34, 53). High mucosal antitoxin IgA antibody concentrations have also been associated with protection against severe or recurrent CDAD (25–27, 51, 56).

Transcutaneous immunization (TCI) involves the needle-free application of antigens directly to hydrated skin from which the stratum corneum has been gently removed (17, 18, 23, 42). TCI usually requires the presence of an immunoadjuvant, and ADP-ribosylating proteins such as cholera toxin (CT) and heat-labile enterotoxin or their derivatives have most commonly been used as immunoadjuvants during TCI (19, 23, 42, 45, 46). During TCI, cutaneously applied antigens are taken up by Langerhans cells in the epidermis, and these cells then migrate to regional lymph nodes. Interestingly, TCI induces both systemic and mucosal immune responses (6, 22, 23, 28, 41, 42, 48). TCI has been shown to be safe and effective in animals and humans (9, 21, 23, 42, 47, 58). In order to assess whether

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TCI would induce immune responses against *C. difficile* toxin A, we therefore transcutaneously immunized mice with a toxoid derivative of *C. difficile* toxin A (CDA), with or without the immunoadjuvant CT, and measured systemic and mucosal anti-CDA immune responses, including induction of toxin A-neutralizing antibodies in immunized mice.

MATERIALS AND METHODS

Preparation of CDA. We purified toxin A from *C. difficile* strain VPI 10463 (American Type Culture Collection, VA) as previously described (35). Briefly, we fractionated culture supernatants by anion-exchange chromatography using a Sepharose column, precipitated toxin A with an acetate buffer, and further purified it by fast protein liquid chromatography using a MonoQ column (Pharmacia, Piscataway, NJ). We inactivated purified toxin A by formalin treatment, using 37% formaldehyde (Sigma Aldrich, St. Louis, MO) at 4°C for 6 days. We dialyzed inactivated CDA overnight at 4°C with regenerated cellulose dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA) against a 100-fold excess of 100 mM phosphate-buffered saline (PBS) with 0.016% formaldehyde and stored the product at 4°C. Prior to use, we concentrated CDA to a final concentration of 1 mg/ml by ultrafiltration through a 50-kDa membrane in a 70-ml concentrator (Amicon, Beverly, MA). We calculated the CDA protein concentration using a bicinchoninic acid assay (Pierce Chemical Company, Rockford, IL), assessed purity by gel electrophoresis, and confirmed decreased toxicity using MRC-5 fibroblast cells in a toxicity assay as described below.

Toxicity assay. To confirm reduced toxicity of CDA, we grew freshly trypsinized MRC-5 cells to confluence in 96-well plates (4×10^4 cells/well) in minimal essential medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum for 5 days at 37°C in a 5% CO_2 atmosphere. We added the CDA preparation to MRC-5 cells starting at 45 μg/well and serially diluted threefold to 0.9 pg/well. We used toxin A as a control. We incubated cells and CDA or wild-type toxin A dilutions at 37°C in a 5% CO_2 atmosphere for 48 h, determining the proportion of cell rounding every 3 h.

Serum neutralization assay. To measure the neutralizing activity of sera, we used MRC-5 cells in a manner similar to that used in the cytotoxicity assay. We incubated twofold dilutions of sera from mice, starting at a 1:50 dilution in minimal essential medium containing 10% fetal bovine serum, at 37°C for 1 h with *C. difficile* toxin A at 60 ng/well. We used four times the minimal dosage of toxin A in the absence of serum required to cause 100% cell rounding after 48 h (0.6-μg/ml final concentration or 60 ng/well). We used commercially available goat anti-*C. difficile* toxin A (List Biological Laboratories, Campbell, CA), toxin A alone, and medium alone as controls. We added toxin-serum mixtures to MRC-5 cells, incubated the plates for 24 h, and determined the proportion of cell rounding. We defined the neutralization antibody titer as the reciprocal of the highest serum dilution that inhibited cell rounding >50%.

Immunization regimen. We immunized female, 3- to 5-week-old, Swiss Webster mice (Taconic, Germantown, NY). Animal work was approved by the Institutional Animal Care and Use Committee. We transcutaneously immunized three cohorts of 15 mice each with either 25 μg of CT (List Biological Laboratories) or 100 μg of CDA or a combination of 25 μg of CT and 100 μg of CDA. We transcutaneously immunized mice on days 0, 14, 28, and 42, as previously described (42). Briefly, we shaved a 3- by 5-cm^2 area on the dorsa of mice by using a clipper with a no. 40 blade (Wahl Clipper Corp, Sterling, IL) and then rested the mice for 24 h. Prior to application of antigen, we anesthetized the mice with 2,2,2-tribromoethanol (Avertin; Sigma Aldrich) administered intraperitoneally at 0.4 mg/g of body weight. We then hydrated the previously shaved area of skin with warm water for 5 min. We then removed the stratum corneum by gently stroking the hydrated area with 10 strokes of an emery board. We then rehydrated the prepared area, applied vaccine antigens, and covered the vaccination site with hydrated gauze and porous Kendall Curity tape (Fisher Scientific, Pittsburgh, PA). The following day, we removed the tape and washed the dorsa of mice with 1 liter of warm water to remove residual antigen. We also immunized a cohort of 15 mice subcutaneously with 25 μg of CDA and 2.5 μg of CT on days 0, 14, 28, and 42.

Immunological sampling. We collected, processed, and stored blood and stool samples from mice on day 0, 12, 26, 40, and 63 as previously described (44). In preparing stool specimens, we placed each stool pellet in 1 ml of a 3:1 mixture of PBS–0.1 M EDTA containing soybean trypsin inhibitor (type II-S; Sigma Aldrich) at a concentration of 0.1 mg/ml and vortexed until the pellet was broken. We centrifuged the mixture twice, added 20 μl of 100 mM phenylmethylsulfonyl fluoride (Sigma) to each 1 ml of final recovered supernatant, and stored samples at −70°C for further analysis.

Measurement of immune responses. To detect antibody responses to CDA, we coated plates with 100 ng/well of purified *C. difficile* toxin A in 50 mM carbonate buffer, pH 9.6. To detect antibody responses to CT, we coated plates sequentially with 1 μg of type III ganglioside (Sigma Aldrich) in 50 mM carbonate buffer (pH 9.6) and then with 100 ng/well of CT in PBS. We blocked plates with PBS–1% bovine serum albumin (BSA) (Sigma Aldrich). To detect anti-CDA and anti-CT IgG and IgA responses in serum, we diluted sera 1:1,000 or 1:50 in PBS containing 0.05% Tween 20 (PBS-T) (Sigma Aldrich), respectively, and incubated the plates at 37°C for 1 h. We detected bound antibodies using a 1:1,000 dilution in PBS-T of either goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) (Southern Biotech, Birmingham, AL) or goat anti-mouse IgA conjugated with HRP (Southern Biotech), incubating plates for 1 h at 37°C. We developed the plates with 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma Aldrich) and 0.03% H_2O_2 (Sigma Aldrich) and determined optical density using a Vmax microplate reader (Molecular Devices Corp, Sunnyvale, CA) at 405 nm kinetically for 5 min at 14-second intervals as previously reported (44). To equilibrate, we divided readings of milliunits of optical density per minute for samples by those for plate controls comprised of pooled blood or stool standards from unrelated experimental cohorts and reported the results as enzyme-linked immunosorbent assay (ELISA) units.

To detect anti-CDA and anti-CT specific antibodies in stool, we first measured total stool IgA. We coated plates with 100 μl/well of rat anti-mouse IgA (Southern Biotech) at a dilution of 1:1,000 in 50 mM carbonate buffer, pH 9.6. Following blocking and washing of plates, we added 100 μl/well of a 1:1,000 PBS-BSA dilution of the previously prepared mouse stool samples and incubated the plates overnight at room temperature. We detected bound antibody using goat anti-mouse IgA-HRP conjugate at a dilution of 1:1,000 in PBS-T–0.1% BSA, incubating plates for 1 h at 37°C. We developed the plates and measured optical density as described above. We calculated total stool IgA using a mouse IgA standard (Kappa TEPC 15; Sigma). To detect specific anti-CDA or anti-CT antibodies in stool, we added 725 μg of total stool IgA in PBS-T to wells in ELISAs as described above.

Statistical analysis. For normally distributed data, we used an unpaired Student *t* test analysis for comparison of means; for nonparametric data, we used the Mann-Whitney U test. We performed statistical analyses using Microsoft Excel 2002 and Statistical Package for Social Sciences (SPSS) version 12.0 and plotted graphs using GraphPad Prism (GraphPad Software, San Diego, CA).

RESULTS

Preparation of CDA. CDA was 46,000 times less toxic than toxin A in a cell-rounding MRC-5 cell assay: after 48 h, toxin A was able to cause cell rounding in a cell-rounding MRC-5 cell assay at a concentration of 0.192 ng/well; after 48 h, formalin-inactivated CDA required 9 μg/well to cause similar cell rounding. Residual formalin was present in the final CDA preparation at 0.016% by volume.

Systemic and mucosal anti-CDA and anti-CT antibody responses in mice transcutaneously immunized with CDA and/or CT. TCI of mice with CDA and CT resulted in a significant anti-CDA IgG response following the second TCI ($P < 0.01$) (Fig. 1A). Mice that were transcutaneously immunized with CDA alone developed a significant serum anti-CDA IgG response following the third immunization ($P < 0.01$). Coadministration of CDA with immunoadjuvantative CT resulted in a significant increase in the serum anti-CDA IgG response by day 63 (following the fourth TCI) in comparison to mice that were transcutaneously immunized with CDA alone ($P < 0.01$). All cohorts of mice that received TCI with CT developed prominent serum anti-CT IgG responses following the first TCI ($P < 0.001$) (Fig. 1B).

Mice that were transcutaneously immunized with CDA and CT developed a significant anti-CDA serum IgA response following the third TCI ($P < 0.05$; Fig. 1C). The concomitant administration of CT during TCI with CDA also resulted in a

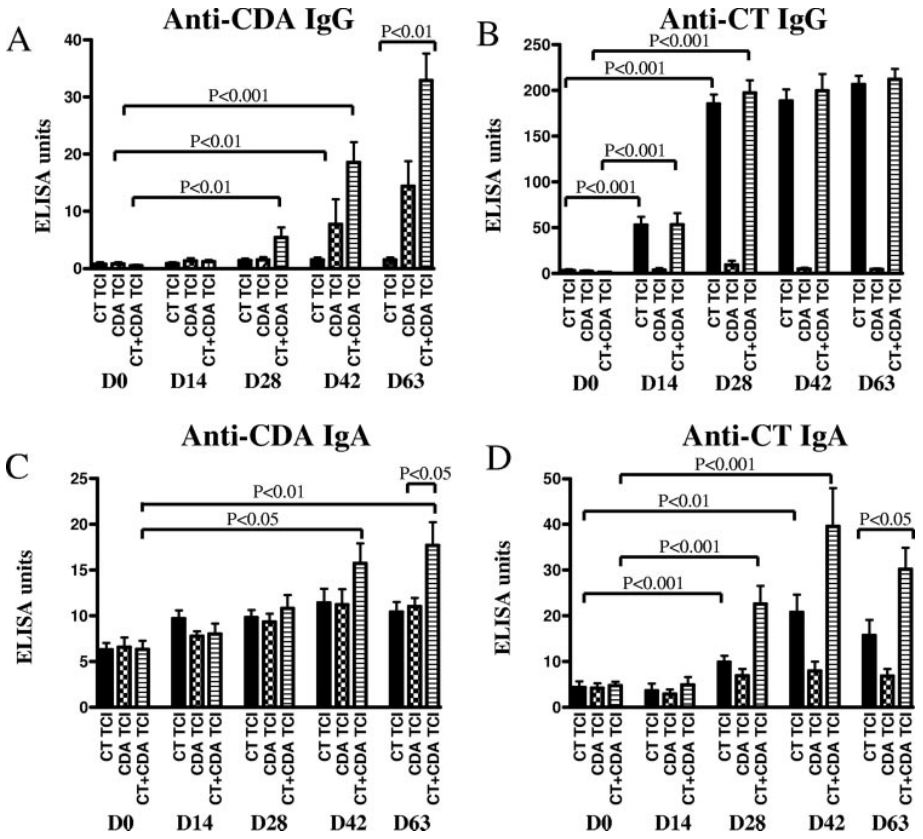

FIG. 1. Serum anti-CDA IgG (A) and anti-CDA IgA (C) responses and serum anti-CT IgG (B) and anti-CT IgA (D) responses in mice transcutaneously immunized on days 0, 14, 28, and 42 with CDA alone (CDA TCI), CT alone (CT TCI), or CT and CDA (CT+CDA TCI). Results were determined by kinetic ELISA and are reported as ELISA units; the geometric mean plus standard error of the mean for each immunization cohort is shown.

significant increase in the anti-CDA IgA serum response in day 63 samples in comparison to the responses in mice that received TCI with CDA alone ($P < 0.05$). Anti-CT serum IgA responses were present in all cohorts of mice transcutaneously immunized with CT following the second TCI ($P < 0.01$) (Fig. 1D).

Comparison of immune responses in mice that were immunized transcutaneously versus responses in mice that were immunized subcutaneously. Comparing responses in day 63 samples by cohorts of animals grouped by route of immunization, mice that were subcutaneously immunized with CDA and CT had a significantly increased serum anti-CDA IgG response in comparison to mice that were transcutaneously immunized with CDA and CT ($P < 0.01$) (Fig. 2A), although anti-CT serum IgG responses were comparable in all mice that were immunized with CT, either transcutaneously or subcutaneously (Fig. 2B). In comparison, mice that were transcutaneously immunized with CDA and CT had a significantly increased day 63 serum anti-CDA IgA response in comparison to mice that were subcutaneously immunized with CDA and CT ($P < 0.05$) (Fig. 2C). Anti-CT IgA serum responses were also significantly increased in mice that were transcutaneously immunized with CDA and CT versus the response in mice that were subcutaneously immunized with CDA and CT ($P < 0.001$) (Fig. 2D).

We also measured immune responses in stool samples. TCI of mice with CDA and CT resulted in a significant anti-CDA IgA response in stool ($P < 0.01$) (Fig. 3A). Interestingly, mice that were transcutaneously immunized with CDA and CT had a significantly increased stool anti-CDA IgA response in comparison to mice that were subcutaneously immunized with CDA and CT ($P < 0.01$). Anti-CT IgA responses in stool were also more prominent in mice that were transcutaneously immunized with CDA and CT than in mice that were transcutaneously immunized with CDA alone ($P < 0.001$) (Fig. 3B) or with CT alone ($P < 0.01$). TCI with CDA and CT also resulted in more prominent stool anti-CT IgA responses than those observed in mice subcutaneously immunized with CDA and CT ($P < 0.001$).

Induction of *C. difficile* toxin A-neutralizing responses. TCI with CDA alone resulted in induction of *C. difficile* toxin A-neutralizing serum antibodies ($P < 0.001$) (Fig. 4). TCI with CDA and immunoadjuvantative CT resulted in an increased toxin A-neutralizing response in comparison to the response seen in mice transcutaneously immunized with CDA alone ($P < 0.001$). Subcutaneous immunization with CDA and CT resulted in the most prominent toxin A-neutralizing response ($P < 0.001$).

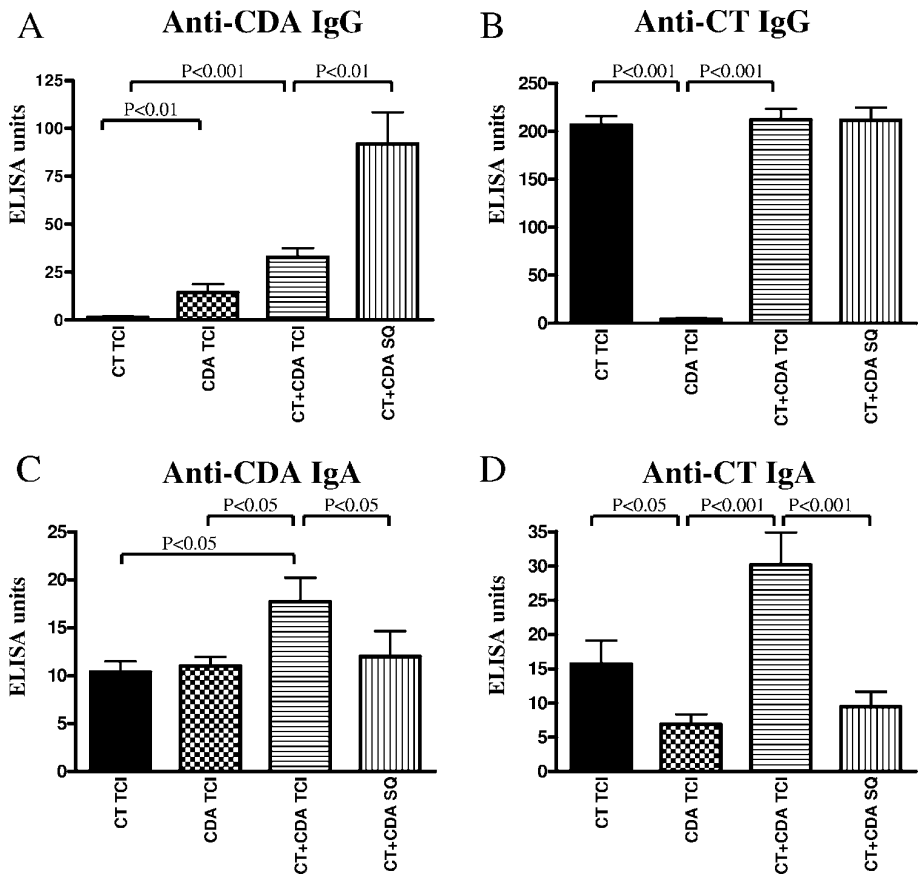

FIG. 2. Day 63 serum anti-CDA IgG (A), anti-CDA IgA (C), anti-CT IgG (B), and anti-CT IgA (D) responses in mice either transcutaneously immunized on days 0, 14, 28, and 42 with CDA alone (CDA TCI), CT alone (CT TCI), or CT and CDA (CT+CDA TCI) or subcutaneously immunized on days 0, 14, 28, and 42 with CT and CDA (CT+CDA SQ). Results were determined by kinetic ELISA and are reported as ELISA units; the geometric mean plus standard error of the mean for each immunization cohort is shown.

DISCUSSION

C. difficile is the leading cause of nosocomial infectious diarrhea, with more than 30% of patients admitted to high-risk hospital wards acquiring *C. difficile* in their intestines and 10% developing CDAD during hospitalization (31). Recently, the emergence of *C. difficile* strain BI/NAP1/r027 has been associated with disease outbreaks, increased severity of CDAD, and CDAD that may be less responsive to treatment (4, 5, 36, 38). Strain BI/NAP1/027 has also been associated with cases of community-acquired CDAD, including cases in individuals who have not recently received treatment with antimicrobial agents. The emergence of BI/NAP1/r027 has been linked to the widespread use of fluoroquinolone antibiotics (14), and increased virulence of strain BI/NAP1/r027 has been attributed to a greater-than-20-fold-increased toxin production compared to that of historical strains (55). Strain BI/NAP1/r027 also expresses a binary toxin whose contribution to virulence is currently unclear (15). Although the spread of *C. difficile* disease can be reduced or prevented by careful adherence to hand hygiene and contact precautions among medical personnel and by isolation of individuals with CDAD, such control practices are costly and have had variable and less-than-optimal results (2, 12, 24), indicating that evaluation of alternative preventative strategies is warranted.

Studies with humans have shown that protection against disease and relapse with *C. difficile* correlates predominantly with the presence of serum antibodies directed against *C. difficile* toxin A and less strongly with anti-toxin B antibody levels (25). Individuals with low anti-toxin A antibody levels are at increased risk of *C. difficile*-associated disease and relapse (33, 34). Studies with humans have also detected anti-toxin A antibodies in intestinal secretions (26), and mucosal anti-toxin A IgA responses contribute to protection against CDAD in animal models (16, 51, 56). Currently, no anti-*C. difficile* vaccine is commercially available, although a candidate vaccine has been evaluated in phase I and IIa studies with humans (1, 29, 49). This vaccine consists of formalin-detoxified *C. difficile* toxins A and B, and parenteral immunizations with this vaccine induce anti-*C. difficile* toxin IgG and toxin-neutralizing antibody responses (1, 29). Since TCI is a noninvasive immunization strategy that induces both systemic and mucosal immune responses, we were interested in evaluating whether TCI with CDA could induce both systemic and mucosal anti-*C. difficile* responses.

We found that TCI with CDA and immunoadjuvantative CT

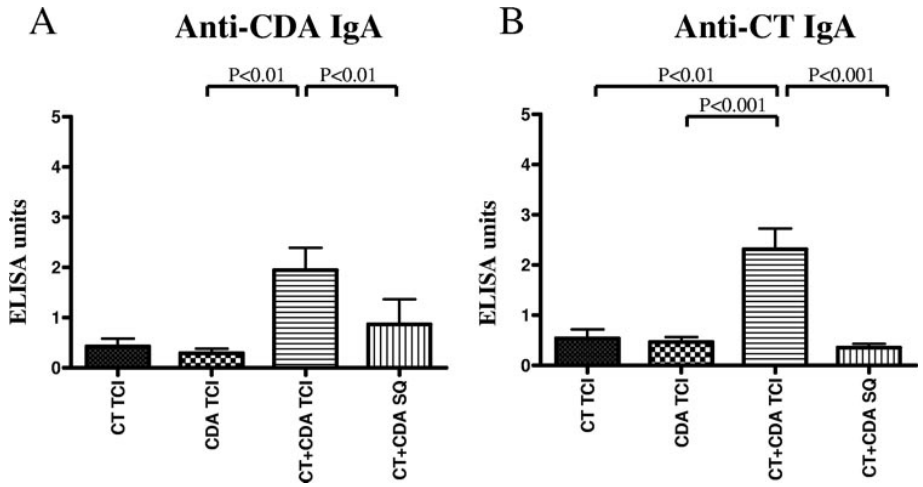

FIG. 3. Day 63 stool anti-CDA IgA (A) and anti-CT IgA (B) responses in mice either transcutaneously immunized on days 0, 14, 28, and 42 with CDA alone (CDA TCI), CT alone (CT TCI), or CT and CDA (CT+CDA TCI) or subcutaneously immunized on days 0, 14, 28s and 42 with CT and CDA (CT+CDA SQ). Results were determined by kinetic ELISA and are reported as ELISA units; the geometric mean plus standard error of the mean each immunization cohort is shown.

induces serum anti-*C. difficile* toxin A IgG responses following two immunizations and induces anti-CT IgG responses following one application. Serum responses against CDA continued to increase following subsequent TCIs, although anti-CT responses were prominent and plateaued following three TCIs. The most prominent serum IgG anti-CDA response occurred in mice that were immunized subcutaneously, although serum anti-CT IgG responses in mice immunized transcutaneously were comparable to responses observed in mice immunized subcutaneously.

Interestingly, parenteral immunization with CDA did not induce serum or stool anti-*C. difficile* toxin A responses, despite repetitive immunization. In comparison, transcutaneous application of CDA with immunoadjuvantative CT resulted in anti-*C. difficile* toxin A in both serum and stool. TCI has previously been shown to induce both mucosal and systemic immune responses (6, 22, 28, 41, 42), including induction of IgA antibody-secreting cell (ASC) responses (20). ASC responses measure transient migration of activated lymphocytes in peripheral circulation prior to lymphocyte homing to mucosal surfaces, and ASC responses correlate with development of subsequent mucosal immune responses at mucosal surfaces (20, 21). The mechanism by which TCI induces mucosal immune responses is currently unclear.

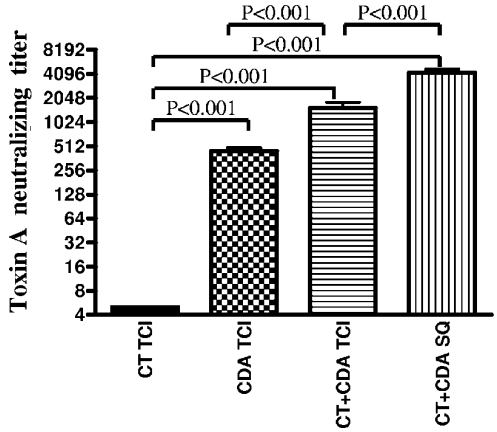

FIG. 4. *C. difficile* toxin A-neutralizing antibody titers in day 63 sera collected from mice transcutaneously immunized with CDA alone (CDA TCI), CT alone (CT TCI), or CT and CDA (CT+CDA TCI) or subcutaneously immunized with CT and CDA (CT+CDA SQ). The neutralizing titer against toxin A was determined by a cell toxicity assay in MRC-5 cells. Results are reported as the geometric mean plus standard error of the mean of the reciprocal titer for each immunization cohort.

Induction of immune responses to antigens applied transcutaneously usually requires coapplication of an immunoadjuvant (19). We found induction of anti-*C. difficile* toxin A IgG and neutralizing antibody responses following TCI with CDA alone, although coadministration of CDA and immunoadjuvantative CT increased the magnitude of the anti-*C. difficile* toxin A IgG and toxin-neutralizing antibody responses. In addition, coadministration of antigen and CT resulted in induction of anti-*C. difficile* toxin A IgA responses in both serum and stool, and such responses were not induced when CDA alone was applied transcutaneously. Mice that were transcutaneously immunized with CT and CDA developed more prominent anti-CT IgA responses in serum and stool than mice that were transcutaneously immunized with CT alone. These observations and our detection of anti-*C. difficile* toxin A responses following TCI with CDA alone may reflect immunoadjuvantative properties of the carboxyl terminus of *C. difficile* toxin A itself (7).

We found that TCI with CDA alone or CDA and CT induced *C. difficile* toxin A-neutralizing antibody responses in serum. Serum *C. difficile* toxin A-neutralizing responses have previously been associated with protection from *C. difficile*-associated disease (16, 29), suggesting that TCI can result in protective anti-*C. difficile* immune responses. The new epi-

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