Distinctive Immunomodulatory and Inflammatory Properties of the *Escherichia coli* Type II Heat-Labile Enterotoxin LT-IIa and Its B Pentamer following Intradermal Administration

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The type I and type II heat-labile enterotoxins (LT-I and LT-II) are strong mucosal adjuvants when they are coadministered with soluble antigens. Nonetheless, data on the parenteral adjuvant activities of LT-II are still limited. Particularly, no previous study has evaluated the adjuvant effects and induced inflammatory reactions of LT-II holotoxins or their B pentameric subunits after delivery via the intradermal (i.d.) route to mice. In the present report, the adjuvant and local skin inflammatory effects of LT-IIa and its B subunit pentamer (LT-IIaB₅) were determined. When coadministered with ovalbumin (OVA), LT-IIa and, to a lesser extent, LT-IIaB₅ exhibited serum IgG adjuvant effects. In addition, LT-IIa but not LT-IIaB₅ induced T cell-specific anti-OVA responses, particularly in respect to induction of antigen-specific cytotoxic CD₈⁺ T cell responses. LT-IIa and LT-IIaB₅ induced differential tissue permeability and local inflammatory reactions after i.d. injection. Of particular interest was the reduced or complete lack of local reactions, such as edema and tissue induration, in mice i.d. inoculated with LT-IIa and LT-IIaB₅ respectively, compared with mice immunized with LT-I. In conclusion, the present results show that LT-IIa and, to a lesser extent, LT-IIaB₅ exert adjuvant effects when they are delivered via the i.d. route. In addition, the low inflammatory effects of LT-IIa and LT-IIaB₅ in comparison to those of LT-I support the usefulness of LT-IIa and LT-IIaB₅ as parenterally delivered vaccine adjuvants.

Cholera toxin (CT) and heat-labile enterotoxins (LTs) produced by *Vibrio cholerae* and by some enterotoxigenic *Escherichia coli* (ETEC) strains, respectively, belong to the family of AB (A,B₅)-type bacterial enterotoxins. The A₅B₅ enterotoxins have an enzymatically active A subunit (28 kDa) that is noncovalently associated to a pentamer composed of five B polypeptides (11.5 kDa each). While the A polypeptide confers the catalytic activity, the B pentamer is responsible for binding to gangliosides and/or galactose-containing surface receptors found on the surface of eukaryotic cells (39). In addition to their roles as virulence determinants directly involved with the watery diarrhea caused by these bacterial pathogens, both CT and LT have received considerable interest due to their remarkable immunomodulatory effects in different mammalian species when they are delivered via parenteral, mucosal, or transcutaneous routes (3, 15, 21, 28, 31, 38, 43).

Based on genetic, biochemical, and immunological features, two major groups of heat-labile enterotoxins have been described (17, 20). Type I enterotoxins produced by ETEC strains share high similarity with *V. cholerae* CT and include the reference LT produced by the H10407 strain (here termed LT-I) while type II enterotoxins (LT-IIa, LT-IIb, and LT-IIc) are usually isolated from ETEC strains derived from nonhuman hosts (16, 33, 37). Although the A peptides of type I and type II enterotoxins are highly homologous, the two toxin groups differ largely in the B subunit amino acid sequences (<14% sequence identity). The different B subunit sequences reflect, at least in part, differential receptor specificity and some distinct immunological features of type I and type II enterotoxins (1, 5, 20). Indeed, previous studies showed that LT-I binds with high affinity to the GM1 ganglioside and, with lower affinity, to a few other galactose-containing surface components such as GM2, asialo-GM1, GD2, GD1b, and paraglobosides while LT-IIa binds most avidly to the GD1b ganglioside and less strongly to other ganglioside receptors (GT1b, GD2, GD1a, GM1, and GM2) (13, 40). Interestingly, the B pentamer of LT-IIa (LT-IIaB₅), but not the holotoxin, interacts with Toll-like receptor 2 (TLR2) to induce secretion of proinflammatory cytokines (19).

Several reports demonstrated that the adjuvant and immunomodulatory properties of LT-IIa correlate with the presence of a functional B subunit (1, 32, 34). Coadministration of LT-IIa and purified antigens via the nasal route promotes enhanced antigen-specific mucosal (IgA) and systemic (IgG) antibody responses and *in vitro* activation of T lymphocytes (31). In contrast, a receptor-binding-deficient mutant of LT-IIa lost most of the immunomodulatory properties (34). Nonetheless, while a plethora of studies have characterized the adjuvant properties of LT-I when it is delivered via parenteral
routes, particularly with the transcytotic (3) and intradermal (i.d.) (44) routes, there are no reports describing the use of type II heat-labile toxins by these routes of administration.

The intradermal immunization route has some advantages over other routes of administration. In comparison with other parenteral routes, considerably smaller amounts of antigen are required to achieve similar immune responses (11, 14). The increased immunogenicity of antigens delivered via the i.d. route may reflect the high density of immune stimulatory cells, such as Langerhans cells and dermal dendritic cells, which are usually resident in the skin (23). In addition, the i.d. immunization route allows easy and prompt assessment of induced local inflammatory effects. However, when delivered via the i.d. route, LT-I induced intense and persistent inflammatory reactions in mice (44). In contrast, no information is available for the inflammatory effects of LT-II enterotoxins, particularly LT-IIa, when they are administered by the i.d. route.

In the present study, using a mouse model, we investigated the adjuvant properties and inflammatory reactions elicited by LT-IIa and LT-IIaB5, delivered via the i.d. administration route. Our results demonstrated that, in comparison to LT-I, LT-IIa and LT-IIaB5, administered by the i.d. route exhibit distinctive immunological and inflammatory properties which may be clinically exploitable.

MATERIALS AND METHODS

Cloning of LT-IIa and LT-IIaB5 subunit-encoding genes. To engineer a His-tagged version of LT-IIa, a fragment encoding the elt operon (including native promoter sequences, corresponding signal sequences, and structural genes) was PCR amplified from the reference LT-IIa-producing ETEC strain SA53 (kindly provided by Beatriz E. C. Guth, Federal University of São Paulo, SP, Brazil) with two specific primers (5'-GGTAGACATGTTGTGGAAAATATAATTTG3'- and 5'-GGTAGATCTCTGACTCTCATTAATCC-3'; restriction sites are underlined). Similarly, to obtain a recombinant version of LT-IIaB5, a fragment encoding the B subunit of LT-IIa, including its signal sequence, was PCR amplified from two specific primers (5'-GCATGGAGAAAGACATGTGCTCT-3' and 5'-CATGATCTCTGACTCTCATTAATCC-3'; restriction sites are underlined). The amplified products were cloned into the NcoI and XhoI sites of the plasmid pQE60, which is an ampicillin-resistant host vector containing the T7 promoter and T7 polymerase. The resulting recombinant pQESDLT-I or pQELT-IIaB5 plasmid encoding LT-IIa or LT-IIaB5, respectively. The resulting recombinant proteins were expressed as B subunit C-terminal His tag fusion proteins.

Purification of the recombinant LTs. LT-I was purified from recombinant E. coli DH5α harboring pM119 (26). Crude protein extracts were resolved by affinity chromatography using immobilized t-galactoside-containing resin (Pierce, Rockford, IL), as previously described (41). LT-IIa and LT-IIaB5 were purified from recombinant E. coli XL1 Blue that had been transformed with pQE60SDLT-Ia and pQE60SDLT-IIaB5, respectively. Cells were cultivated at 37°C with vigorous shaking (200 rpm) in Luria-Bertani broth supplemented with ampicillin (100 µg/ml). Expression of the recombinant proteins was achieved by addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 0.3 mM) to mid-log-phase cells (optical density [OD] of ∼0.5 to 0.6). After 4 h of incubation, cells were harvested, suspended in phosphate-buff ered saline (PBS; pH 7.4), and disrupted in a high-pressure homogenizer at a pressure of 6,000 lb/in2 (Arpeçás, Brazil). Insoluble cell debris was removed by centrifugation at 10,000 x g for 30 min at 4°C. Recombinant LT-IIa and LT-IIaB5 were purified by means of affinity chromatography using nickel-nitrilotriacetic acid-containing resin (GE Healthcare, Sweden). In addition, size exclusion chromato graphy performed in a HiLoad 16/60 Superdex 75 prep-grade column (Amersham Biosciences, United Kingdom) was employed to separate the holotoxin from residual amounts of B subunit contaminants. All purification procedures were performed using an AKTA fast protein liquid chromatography (FPLC) system (GE Healthcare). The purity of the recombinant proteins was confirmed by SDS-PAGE and immunoblots. The purity of the recombinant proteins was confirmed by SDS-PAGE and immunoblots using rabbit polyclonal antibodies generated against LT-IIa. Protein concentration was determined spectrophotometrically using the Edelshoeb method (10) according to the following formula: concentration (mg/ml) = (absorbance at 280 nm x 280 nm) / molecular mass (Da), where x 280 nm represents number of tryptophans x 5,690 + number of tyrosines x 1,280. The level of contaminating endotoxin was determined in each recombinant protein sample by the Limulus amebocyte lysate method (Lonza, Walkersville, MD). The endotoxin amounts were lower than 3,000 endotoxin units (EU)/mg of purified protein, which corresponds to ≤1.5 endotoxin units per dose.

Intracellular cAMP concentration. Mouse I-A+ dendritic cells were maintained in Dulbecco's modified Eagle's medium (DMEM; CultiLab, Brazil) supplemented with 10% fetal bovine serum ( Gibco Laboratories, Grand Island, NY). Ninety-six-well round-bottom plates were seeded with 5 x 104 cells/well cultivated to near confluence at 37°C in 5% CO2-containing atmosphere. One microgram of each toxin was treated with 10 ng of trypsin in TEAN (50 mM Tris-HCl, 1 mM EDTA, 3 mM NaN3, and 0.2 M NaCl, pH 7.4) at 37°C for 45 min and then added to each well. At 1, 2, and 3 h after toxin addition, cells were submitted to lysis, and the cyclic AMP (cAMP) concentration was measured with an Amersham CAMP enzyme immunoassay (ELIA) system (Amersham Life Science, United Kingdom). The cAMP concentrations (pmol/ml) were calculated according to the manufacturer's instructions.

GD1b-binding assay. Binding of LT-IIa and LT-IIaB5 to the GD1b ganglioside receptor was performed as previously described (36) with some modifications. Briefly, polyvinyl 96-well enzyme-linked immunosorbent assay (ELISA) Polysorp plates (Nunc, Roskilde, Denmark) were coated overnight at room temperature with 2 µg/ml GD1b (Sigma Aldrich, St. Louis, MO) in PBS (100 µl/well). After washings and a blocking step with 200 µl/well of 0.1% bovine serum albumin (BSA) in PBS containing 0.05% Tween (PBS-T-0.1% BSA), aliquots (cor responding to 1.2 µM) of native or heat-denatured (100°C for 30 min) LT-IIa and LT-IIaB5 were added to wells in a final volume of 100 µl and subsequently serially diluted. Plates were incubated for 2 h at room temperature, washed, and added with 100 µl/well of rabbit anti-LT-IIa hyperimmune serum (diluted 1:500 in PBS-T-0.1% BSA). Plates were incubated for another 1.5 h at room temperature, washed, and reacted with PBS-T-0.1% BSA-diluted (1:3,000) horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma Aldrich) incubated for 90 min at room temperature. After a final washing step, color reactions were developed with o-phenylenediamine and H2O2. After 20 min at room temperature, the reactions were interrupted by the addition of 2 M H2SO4 (50 µl/well), and the OD at 492 nm (OD492) was measured in a microplate reader (Multiskan EX, ThermoLabSystems, Finland).

Mice intradermal immunizations. Female C57BL/6 mice aged 8 to 12 weeks were obtained from the Isogenic Mouse Breeding Facility of the Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo (USP). All animal experiments were approved by the laboratory's Animal Care and Ethical Use Committee from the Institute of Biomedical Science at USP, in accordance with Brazilian code. Animals were subjected to light anesthesia with a solution containing 75 mg/kg of ketamine and 10 mg/kg of xylazine, shaved on the dorsum, and rested for 48 h before each immunization procedure. Mice were i.d. inoculated with 20-µl aliquots containing saline (negative control), 100 µg of ovalbumin (OVA) diluted in saline (Sigma Chemical Co., St. Louis, MO), or OVA diluted in saline containing 0.5 µg of LT-I, LT-IIa, or LT-IIaB5. The intradermal immunization regimen consisted of three doses delivered on consecutive days. Blood was obtained from the retro-orbital plexus 1 day before each inoculation (days 0, 14, and 28) and 2 weeks after the last dose (day 42). Sera obtained from the blood were stored at −20°C. Two weeks after the last immunization, inguinal draining lymph nodes (DLNs) were excised, processed, and immediately pooled for enzyme-linked immunosupopt (ELISPOT) assays. Spleens were surgically removed and processed, and cells were either immediately pooled for cell culture assays or treated individually for ELISPOT assay and for in vivo cytotoxic CD8+ T lymphocyte analysis.

Quantification of antibodies by ELISA. Anti-enterotoxin and anti-OVA serum IgG titers were determined by conventional ELISA (12). Serum anti-enterotoxin IgG and anti-OVA IgG titers were measured using polyvinyl 96-well enzyme-linked immunosorbent assay plates (Nunc) that had been coated with LT-I, LT-IIa, or LT-IIaB5. The immunoprecipitation regimen consisted of three doses administered on consecutive days. Blood was obtained from the retro-orbital plexus 1 day before each inoculation (days 0, 14, and 28) and 2 weeks after the last dose (day 42). Sera obtained from the blood were stored at −20°C. Two weeks after the last immunization, inguinal draining lymph nodes (DLNs) were excised, processed, and immediately pooled for enzyme-linked immunosupopt (ELISPOT) assays. Spleens were surgically removed and processed, and cells were either immediately pooled for cell culture assays or treated individually for ELISPOT assay and for in vivo cytotoxic CD8+ T lymphocyte analysis.

Secreted cytokine measurements. For evaluation of cytokine production, spleen cells were harvested from animals euthanized 2 weeks after the immunization protocol (44). Spleen cells from mice of the same immunization group were pooled (1 x 107 viable cells/ml) and cultured in a CO2-containing atmosphere at 37°C in the presence or absence of OVA (5 µg/ml), the OVA class I (Kβ)-restricted CD8+ T cell epothe synthetic peptide (25-35)SINFEKL-26, here
FIG. 1. Purification and biological activities of LT-IIa and LT-IIaB5. (A) SDS-PAGE of LT-IIa and LT-IIaB5 purified by nickel affinity chromatography. Lane 1, LT-IIa (2.5 μg); lane 2, LT-IIaB5 (1.7 μg); M, PageRuler Unstained Protein Ladder (Fermentas). (B) Kinetic of cAMP accumulation in Y-1 cells exposed to trypsin-activated LT-I (○) or LT-IIa (●). One representative of three experiments is shown. Values are indicated as means ± standard errors of the means. (C) GD1b-binding of recombinant LT-IIa and LT-IIaB5. Equimolar amounts of purified LT-IIa (●) and LT-IIaB5 (○) were incubated under non-denaturing conditions or LT-IIaB5 (△) and LT-IIaB (■) after incubation at 100°C for 30 min were tested for GD1b-binding by ELISA. One representative of two experiments is shown. Values are indicated as means ± standard deviations.

In vivo cytotoxic activity of CD8+ T lymphocytes. The in vivo cytotoxic activity of CD8+ T cells in OVA257-264-pulsed spleen cells was determined (2). Splenocytes from naive mice were labeled with 0.5 μM or 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen Corp., Carlsbad, CA) for 15 min at 37°C. The cell population stained with 5 μM CFSE was incubated with the OVA257-264 (SIINFEKL) peptide for 40 min at 37°C. After this period, both cell populations (0.5 μM CFSE-labeled and 5 μM CFSE- and OVA257-264-labeled cells) were intravenously (i.v.) injected (2 × 106 cells of each preparation) into immunized mice. On the following day, the inoculated animals were euthanized, and individual spleen cells were collected and homogenized for isolation of single cells. The percentages of specific-cell killing were calculated by the relative reduction of peptide-pulsed cells relative to the nonpulsed cells (7).

Skin permeability test. To evaluate the degree of skin permeability induced by i.d. administered enterotoxins, the rate of diffusion of blue dextran (mass, 200,000 Da) (Sigma Aldrich) or 0.5 μM of rat anti-mouse monoclonal antibody (MAb) specific to IFN-γ (BD Biosciences Pharmingen) into the dermis. Sections placed on glass slides were stained with hematoxylin and eosin. Stained slides were inspected under an inverted microscope (Axiovert 200; Zeiss, Germany), and representative fields were photographed.

Statistical evaluations. Data were compared using analysis of variance (ANOVA) followed by a Tukey test. P values of <0.05 were considered to be indicative of statistical significance.

RESULTS

Purification and in vitro characterization of the recombinant LT-IIa and LT-IIaB5. Recombinant LT-IIa and LT-IIaB5 were engineered with histidine tags at the carboxyl end of the B monomers. The recombinant LT-IIa and LT-IIaB5 were recovered in apparent homogeneity (Fig. 1A) with preserved antigenicity, as demonstrated by reactivity with rabbit polyclonal antibodies generated with LT-IIa (data not shown). Functional analyses of the recombinant proteins were carried out by cAMP accumulation in Y-1 cells and GD1b-binding experiments. Trypsin-activated LT-IIa and LT-I showed similar cAMP-inducing activity (Fig. 1B). As expected, only residual cAMP-inducing activity was detected in cells incubated with LT-IIaB (data not shown). Similarly, recombinant LT-IIa and LT-IIaB5 bound to GD1b-coated plates with similar affinities while samples boiled for 30 min did not exhibit detectable GD1b-binding activity (Fig. 1C).

Immunogenicity and humoral adjuvant effects of intradermally delivered LT-IIa and LT-IIaB5. To evaluate the i.d. adjuvanticity of LT-IIa and LT-IIaB5, C57BL/6 mice were immunized and boosted twice with 100 μg of OVA in the presence of LT-IIa or LT-IIaB5 (0.5 μg/dose). Anti-OVA (adjuvanticity) and anti-LT (immunogenicity) serum antibody responses were measured 2 weeks after each immunization dose. As a positive control, a mouse group was immunized with OVA in the presence of LT-1 (0.5 μg/dose). After the primary immunization and first boost, anti-LT-I serum IgG titers were significantly higher than the anti-LT-
saline (sham), OVA, or OVA plus LT-I, LT-IIa, or LT-IIaB5 2 weeks independently performed experiments. Individual anti-LT (C) and serum samples of each immunization group (n = 1246 MATHIAS-SANTOS ET AL. CLIN. VACCINE IMMUNOL.

At day 42 in mice submitted to the complete immunization values represent means ± standard errors of the means of individual serum samples of each immunization group (n = 5) in one of two independently performed experiments. Individual anti-LT (C) and anti-OVA (D) serum IgG titers were detected in mice immunized with saline (sham), OVA, or OVA plus LT-I, LT-IIa, or LT-IIaB5, 2 weeks after the third dose (day 42). Data are based on two separate immunization tests (n = 10). The bars represent the mean antibody titers detected in sera of animals subjected to the different immunization regimens. Brackets indicate pair comparison of statistically significant differences observed among the tested immunization groups. *, P < 0.05.

IIa and anti-LT-IIaB5 serum IgG titers (Fig. 1A). After the second boost, the serum anti-LT IgG titers in mice immunized with LT-I were elevated in comparison to the titers of mice immunized solely with LT-IIa. Yet both values differed significantly from the anti-LT serum IgG titers detected in mice immunized with either LT-I or LT-IIaB5 (Fig. 2A). A similar secretion pattern was also observed when the IL-10 levels were determined in cells collected from mice submitted to the different immunization regimens (Fig. 3D). On the other hand, the levels of secreted IL-5 were relatively higher in cultures of cells derived from mice immunized with OVA in combination with LT-I or with OVA in combination with LT-IIaB5 (Fig. 3C).

To determine the T cell populations mainly involved in cytokine secretion following incubation with the target antigen, spleen cells collected from immunized mice were incubated with synthetic peptides corresponding to the dominant major histocompatibility complex class I (MHC-I)-restricted T CD8+ epitope (OVA257–264) or the MHC-II-restricted T CD4+ epitope (OVA265–286) recognized by C57BL/6 mice. The data demonstrated that IFN-γ was mainly produced by cells incubated with the OVA257–264 peptide. IL-5, however, was preferentially secreted by cells following stimulation with the OVA265–280 peptide (Fig. 3B and C). Finally, smaller amounts of IL-10 were detected in culture supernatants of cells incubated with either OVA265–280 or OVA257–264 peptide than in culture supernatants of cells incubated with only purified OVA (Fig. 3C and D). These results indicated that LT-I, LT-IIa, and, to a lesser extent, LT-IIaB5, activate secretion of both Th1- and Th2-associated cytokines produced commonly by CD4+ T cells and by CD8+ T cells.

**Activation of OVA-specific cytotoxic CD8+ T cell responses.**

Activation of antigen-specific cytotoxic CD8+ T cell responses was measured in mice that had been i.d. immunized with LT-IIa or LT-IIaB5. The total numbers of IFN-γ-secreting CD8+ T cells derived from spleens or inguinal draining lymph nodes (DLNs) were determined following in vitro stimulation with the OVA257–264 peptide. LT-I and LT-IIa, but not LT-IIaB5, induced similar activation patterns of splenic and DLN-derived CD8+ T cells as measured following stimulation with OVA257–264 (Fig. 4A). As an alternative approach to confirm the T cell adjuvant effects of i.d. delivered LT-IIa or LT-IIaB5, we determined the in vivo cytotoxic activity of activated OVA-specific CD8+ T cells. Briefly, mice submitted to the different immunization regimens were i.v. administered with naive spleen cells fluorescently labeled and either untreated or pre-incubated with the OVA257–264 peptide. After 18 h, the pres-

**FIG. 2.** Immunogenicity and adjuvant effects of LT-I, LT-IIa, and LT-IIaB5 after i.d. administration to mice. C57BL/6 mice were immunized with saline (sham), OVA (100 μg), or OVA combined with 0.5 μg of LT-I, LT-IIa, or LT-IIaB5 as vaccine adjuvants on days 0, 14, and 28. Individual serum samples were collected 2 weeks after each immunization dose and tested for anti-LT (A) and anti-OVA (B) serum IgG titers.

Intradermal administration of OVA and LT-I or LT-IIa revealed clear adjuvant effects on the induced serum anti-OVA IgG responses with regard to mice immunized only with OVA (Fig. 2B). Mice immunized with OVA and LT-IIaB5 elicited enhanced serum anti-OVA IgG titers after the first and second i.d. doses. The differences, however, did not attain statistical significance after the third dose. Analysis of individual results and inclusion of a larger number of immunized animals revealed that, after the second boosting dose, mice immunized with LT-IIaB5 showed variable results, and approximately 70% of the vaccinated mice elicited enhanced anti-OVA IgG responses in comparison to mice immunized with the antigen without adjuvant (Fig. 2D).

**T cell adjuvant effects.** To determine whether the i.d. administration of LT-IIa or LT-IIaB5 would enhance T cell responses, spleen cells collected from vaccinated mice and in vitro stimulated with purified OVA were evaluated for secretion of specific cytokines. Determination of the IFN-γ/IL-5 ratios suggested that mice immunized with LT-I or LT-IIa elicited a mixed Th1/Th2 response pattern with values of 10.4 and 45.6, respectively, while mice immunized with LT-IIaB5 showed a lower ratio (2.2) (Fig. 3A). The amounts of IFN-γ secreted by cells from mice immunized with OVA plus LT-I or LT-IIa were higher than the amount detected in mice immunized with OVA alone or with OVA in combination with LT-IIaB5 (Fig. 3A). A similar secretion pattern was also observed when the IL-10 levels were determined in cells collected from mice submitted to the different immunization regimens (Fig. 3D). On the other hand, the levels of secreted IL-5 were relatively higher in cultures of cells derived from mice immunized with OVA in combination with LT-I or with OVA in combination with LT-IIaB5 (Fig. 3C).
ence of unpulsed and peptide-pulsed target cells was measured in the spleens of individual mice using cytometer analyses. Clearly, mice that were immunized with LT-I (56% specific cell lysis) or LT-IIa (59% specific cell lysis) mounted statistically significantly elevated CD8$^+$ T cell cytotoxic responses in comparison to mice immunized only with OVA or to mice coimmunized with OVA and LT-IIaB5 (Fig. 4B). These results demonstrated that LT-IIa, but not LT-IIaB5, exhibited strong CD8$^+$ T cell adjuvant effects following i.d. administration in the mouse model.

Skin permeability and edema. Tissue permeability effects induced by CT and LT, particularly at mucosal epithelia, have been reported to have a relevant role in the observed adjuvant properties of these two enterotoxins (22, 30, 42). To determine if the distinct adjuvant effects of LT-IIa and LT-IIaB5 could be explained, at least in part, by effects on skin permeability, mice were i.d. injected with a fixed amount (0.5 μg) of either LT-I, LT-IIa, or LT-IIaB5 that had been admixed with dextran blue, a high-molecular-weight glucose polymer-containing dye that does not commonly diffuse into the dermis. In contrast to mice inoculated only with dextran blue, mice inoculated with LT-I or LT-IIa displayed a clear diffusion of the dye around the injection site. Animals injected with LT-IIa, however, exhibited a larger amount of diffusion of the dye at time points of 10 h and 30 h. In contrast, no tissue permeability effects were evident in mice inoculated with LT-IIaB5 (Fig. 5A).

It was deemed feasible that a small amount of LPS in the purified enterotoxins could have elicited the effects on diffusion. Thus, to determine if the presence of residual LPS induced similar effects, the experiments were repeated using purified Salmonella Enteriditis LPS at a level that was 4-fold higher in concentration than that measured in the LT preparations containing the largest amount of LPS contamination. No dye diffusion was observed in mice treated with purified LPS (Fig. 5A). Furthermore, mice treated with heat-treated LT-I and LT-IIa failed to induce permeability effects, thus providing more evidence that the effects on permeability by the enterotoxins was not stimulated by residual LPS in the stocks.
These results indicated that both LT-I and LT-IIa have skin permeability effects when they are i.d. administered in the mouse model. LT-I or CT induces strong inflammatory reactions when administered via the i.d. route (11, 44). To determine if LT-IIa or LT-IIaB5 had the capacity to induce similar inflammatory reactions, mice were i.d. administered with the toxins, and the amounts of edema and self-limited induration were evaluated at the site of inoculation (Fig. 5B). Animals injected with LT-IIa developed mild inflammatory effects. In contrast, mice receiving LT-IIaB5 developed no visible symptoms of inflammation (Fig. 5B). The mild swelling (with no visible erythema and less induration) observed in mice receiving LT-IIa vanished after 4 days (Fig. 5B). Mice i.d. inoculated with LT-I, however, developed a strong inflammatory reaction at the site of inoculation, including development of extensive edema and local induration that persisted for at least 7 days (Fig. 5B). Administration of purified LPS at a concentration at least 4-fold higher than concentrations detected in the LT preparations did not induce detectable inflammatory reactions (Fig. 5B). Similarly, mice treated with boiled LT preparations did not develop inflammation symptoms (data not shown). Incu-

FIG. 4. Activation of CD8\(^+\) T cell responses in mice i.d. immunized with OVA and LT-I, LT-IIa, or LT-IIaB5. (A) Cells collected from spleens (SP) and pooled inguinal DLNs from mice i.d. immunized with three doses of OVA alone (white bars) or coadministered with LT-I, LT-IIa, or LT-IIaB5 were \textit{in vitro} stimulated 24 h in the presence of the MHC-I-restricted CD8\(^+\) T cell-specific peptide (OVA\(_{257-264}\)). The numbers of IFN-\(\gamma\)-secerting cells were determined by ELISPOT assay. Values represent means \(\pm\) standard errors of the means. Data are representative of two independent experiments. (B) \textit{In vivo} cytotoxic activity of OVA-specific activated CD8\(^+\) T cells generated in mice subjected to the different i.d. immunization regimens. Two weeks after the last dose, immunized mice were i.v. injected with OVA\(_{257-264}\)-pulsed and nonpulsed CFSE-stained spleen cells from naïve mice. The \textit{in vivo} cytotoxic activity induced in immunized mice was determined based on the reduction in the number of OVA\(_{257-264}\)-pulsed cells with regard to cells not pulsed with the peptide as described in the Materials and Methods section. The results represent the percentage of specific cell lysis observed 24 h after the i.v. administration of the CFSE-stained cells. The experiment was repeated three times, and data represent mean \(\pm\) standard deviations of each immunization group (\(n = 5\)). Significantly different values (\(P < 0.05\)) with regard to mice immunized with OVA alone are indicated (#).

FIG. 5. Skin permeability and swelling reaction observed in mice i.d. inoculated with purified LPS, LT-I, LT-IIa, or LT-IIaB5. (A) Skin permeability was measured after the i.d. injection of dextran blue (12.5\% wt/vol) alone or admixed with 6.0 EU of purified LPS or with 0.5 \(\mu\)g of LT-I, LT-IIa, or LT-IIaB5. Dye diffusion was measured every 10 h in the course of 2 days after administration. Significantly different values (\(P < 0.05\)) between mice i.d. injected with LT-I and LT-IIa are indicated (#). (B) Edematous i.d. reaction after injection of saline (sham), LPS (6.0 EU), or each purified protein (0.5 \(\mu\)g). Swelling of the injection site was measured periodically up to 4 days after injection. Symbols are identified on the figure. (C) Edematous i.d. reaction in mice inoculated with LT-I or LT-IIa previously either untreated or incubated with specific anti-LT sera or the cognate ganglioside receptors (GM1 for LT-I or GD1b for LT-IIa). Edema sizes were measured 48 h after administration. Data represent means \(\pm\) standard deviations of 4 to 5 mice under each tested condition.
bation of LT-I with purified GM1 ganglioside completely ablated the inflammatory reaction (44). When LT-IIa was preincubated with GD1b, no significant decrease in the local inflammatory reactions was observed (Fig. 5C).

Experiments were also designed to determine if the presence of anti-LT antibodies could neutralize the inflammatory reactions observed in mice inoculated with either LT-I or LT-IIa. Incubation of LT-I or LT-IIa with specific polyclonal antiserum did not reduce the extent of edema noted in the enterotoxin-administered mice (Fig. 5C). Similarly, no reduction in the inflammatory effects was observed in mice that received multiple immunizations with either LT or LT-I (data not shown). To more precisely describe the inflammatory effects induced (or not induced) by i.d. administration of the enterotoxins, skin biopsy specimens were prepared. Samples of skin located proximal to the site of inoculation were obtained at a point 2 days after administration of LT-I, LT-IIa, or LT-IIaB5 (Fig. 6). Intense local infiltration of polymorphonuclear cells throughout the epidermis, dermis, and subcutaneous tissues was observed in skin samples collected from mice injected with LT-I (Fig. 6B). In contrast, much less cellular infiltration was noted in the skin samples of mice that had received LT-IIa (Fig. 6C). No significant histological alterations were observed in samples collected from mice inoculated with LT-IIaB5.

**DISCUSSION**

While a variety of studies demonstrated the potent immunomodulatory properties of the type II enterotoxins used as mucosal adjuvants (31, 34, 35), the capacity of these enterotoxins to enhance immune responses to coadministered antigens following administration via the i.d. route had not been evaluated. Results described here show that LT-IIa displays humoral and cellular adjuvant effects similar to those of LT-I following i.d. administration to C57BL/6 mice. Yet in contrast to LT-I, LT-IIa induces a significantly lower inflammatory reaction at the injection site, as evidenced by reduced edema formation, less persistent induration, and decreased inflammatory cellular infiltrates. In addition, mice i.d. injected with LT-IIaB5 exhibited an increased antigen-specific serum IgG response although neither enhancements in T cell responses nor detectable inflammatory reactions at the injection site were observed. Our results demonstrate, therefore, that LT-IIa and LT-IIaB5 show distinct inflammatory effects with regard to LT-I following i.d. administration to mice.

LT-IIa and LT-IIaB5 showed differential parenteral adjuvanticity in a mouse model. C57BL/6 mice i.d. immunized with OVA admixed with LT-I or LT-IIa exhibited similar enhancement of antigen-specific serum antibody responses and T cell activation patterns, including induction of cytotoxic CD8+ T cells. On the other hand, mice i.d. immunized with LT-IIaB5 elicited only lower anti-OVA antibody titers, which did not reach statistically significant increments following two boosts with regard to mice immunized with OVA. These results contrast with the in vitro results that indicated that LT-IIaB5 has stronger immunomodulatory activities than those associated with holotoxin (18, 19). Results based on the responses of human monocyct cells indicated that LT-IIaB5 induces stronger in vitro proinflammatory activity, as measured by secretion of cytokines either alone or in combination with LPS, than cells exposed to LT-IIa (18, 19). The differential in vitro immunomodulatory activity of LT-IIa and its B subunit pentamer is thought to mainly correlate with the binding of LT-IIaB5 to the TLR2 innate immune receptor through a domain not exposed at the holotoxin (19, 27). Our results show that activation of innate immunity responses mediated by the B subunit does not contribute to the in vivo effects of LT-IIa, whereas the presence of an intact A subunit has a relevant role in the in vivo inflammatory and adjuvant effects of the enterotoxin. In fact, it would appear that multiple factors are involved in vivo immunological properties of LT-IIa and LT-IIaB5 that likely include binding and activation of antigen-presenting cells, stimulation of innate immunity signals leading to induction of adaptive immune responses, and/or species-specific genetic features that are still not fully elucidated either for LT-I or LT-IIa. Clearly, additional studies are needed to fully elucidate the mechanisms underlying the immune-modulatory properties of these enterotoxins, particularly those elicited by parenteral administration.

Induction of cellular immune responses is a crucial feature for eradication of intracellular pathogens and the control of certain degenerative diseases. Mice immunized with LT-IIa or LT-I showed similar T-cell activation patterns, as demonstrated by the secreted cytokine profiles and activation of cytotoxic CD8+ T cells. In contrast to the antibody-inducing property, in which LT-IIaB5 retained at least partial inducing activity, mice immunized with the B subunit pentamer showed greatly reduced activation of antigen-specific T cells, which was mainly restricted to IL-5 secretion by T helper cells, with regard to LT-IIa. These results further indicate that the presence of an intact A subunit is important for the T cell adjuvant effects of LT-IIa, particularly for activation of cytotoxic CD8+ T cell responses following i.d. administration to mice. In accordance with previous in vitro results (1, 18, 19, 28), our data...
support the use of LT-IIa as a potent parenteral adjuvant for T cell responses and, therefore, a promising alternative as i.d. adjuvant for vaccines targeting chronic infections by intracellular pathogens as well as tumor cells.

Tissue permeability activity of type I enterotoxins has been reported as a putative feature involved with the adjuvant properties of these toxins (22, 29, 30, 42). Based on a method originally designed to measure the vascular skin permeability effect of CT (9), we tested the skin permeability effects of LT-I and LT-IIa by simply i.d. injecting these toxins with dextran blue, a high-molecular-weight dye that does not diffuse away from the injection site. Using this methodological approach, we observed that both LT-I and LT-IIa induced similar effects on skin permeability while no effects were observed in mice treated with LT-IIaB5. Notably, administration of neither purified LPS at a higher concentration than that found in the purified LTs nor heat-denatured toxins elicited effects on skin permeability. Compared with LT-IIa, however, LT-I induced much stronger inflammatory reactions at the injection site. As previously reported (44), and as confirmed by our own observations, mice i.d. inoculated with LT-I showed increased redness, local swelling, and induration that persisted for several days. In contrast, symptoms detected in mice treated with LT-IIa were significantly milder and short-lived while no local inflammatory reactions were detected in mice i.d. inoculated with LT-IIaB5. These results show that the skin inflammatory effect is related to the adjuvant activities of both LT-I and LT-IIa following i.d. administration to mice. In contrast, the strong local inflammatory reactions caused by LT-I are clearly related to the ability of the GMI-binding activity of the toxin while binding to GD1b seems to play a minor role in the effect exerted by LT-IIa. Interestingly, the presence of anti-LT antibodies did not appear to affect the i.d. reactions observed in mice treated with LT-I or LT-IIa. These results suggest that induction of inflammatory local reactions does not correlate with the adjuvant activities of either LT-I or LT-IIa. Further experiments based on toxin mutants altered either at the catalytic site or at residues affecting the receptor-binding specificity will contribute to the elucidation of the specific roles of the A and B subunits on the induction of the local inflammatory reactions associated with type I and type II LTs.

It is generally accepted that a local acute inflammatory response observed after parenteral vaccination is essential for evoking a strong primary antigen-specific immune response (4, 24), an effect that is likely ascribed to the cross talk of the various immunocompetent cell types that are recruited to the inoculation site. Due to the multiple cellular interactions, activation of internal regulatory circuits, and production of immunomodulatory molecules, local inflammatory reactions are usually induced by vaccines administered via parenteral routes. In extreme cases, acute inflammatory responses evoked by vaccination lead to allergic reactions, autoimmune disease, anaphylactic shock, and other undesirable outcomes (6, 25). In addition, when applied via the nasal mucosal route, LT-I has raised considerable concern regarding translocation of the toxin and coadministered antigens to the central nervous system, increasing the risks of neurotoxicity (8). It is notable, therefore, that when introduced by the parenteral route, LT-IIa, although expressing similar adjuvant properties to LT-I, induced a milder local inflammatory reaction. Even the modest increase in the antibody responses elicited in mice immunized with LT-IIaB5 occurred without significant induction of local inflammatory reactions, thus suggesting that development of LT derivatives devoid of any inflammatory activity or development of derivatives unable to elicit undesirable effects is a feasible goal. In this context, the present results shed new light on the use of LT-IIa as an alternative for the development of parenteral adjuvants.

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