Type II heat-labile enterotoxins (HLTs) constitute a promising set of adjuvants that have been shown to enhance humoral and cellular immune responses when coadministered with an array of different proteins, including several pathogen-associated antigens. However, the adjuvant activities of the four best-studied HLTs, LT-IIa, LT-IIb, LT-IIbT13I, and LT-IIc, have never been compared side by side. We therefore conducted immunization studies in which LT-IIa, LT-IIb, LT-IIbT13I, and LT-IIc were coadministered by the intradermal route to mice with two clinically relevant protein subunit vaccine antigens derived from the enzymatic A subunit (RTA) of ricin toxin, RiVax and RVEc. The HLTs were tested with low and high doses of antigen and were assessed for their abilities to stimulate antigen-specific serum IgG titers, ricin toxin-neutralizing activity (TNA), and protective immunity. We found that all HLTs tested were effective adjuvants when coadministered with RiVax or RVEc. LT-IIa was of particular interest because as little as 0.03 μg when coadministered with RiVax or RVEc proved effective at augmenting ricin toxin-specific serum antibody titers with nominal evidence of local inflammation. Collectively, these results justify the need for further studies into the mechanism(s) underlying LT-IIa adjuvant activity, with the long-term goal of evaluating LT-IIa’s activity in humans.
by side in a mouse intraderal (i.d.) immunization model. We chose to use RiVax and RVEc for these studies, because they are each well-characterized investigational vaccine antigens that have been deemed safe in phase I clinical trials (4, 5). Both are recombinant, nontoxic derivatives of ricin’s enzymatic subunit, RTA. RiVax is a full-length (267-residue) variant of RTA with two attenuating point mutations at residues Y80 and V76, while RVEc lacks RTA’s C terminus (residues 199 to 267) as well as a small hydrophobic loop in the N terminus (residues 34 to 43). Despite their different physical makeups, RiVax and RVEc were reported to be indistinguishable in terms of stimulating protective immunity to ricin in mice (12). Moreover, we have previously examined the potential of LT-IIb and LT-IIIbT13I to serve as adjuvants for RiVax when administered to mice via the intraderal (i.d.) and intranasal (i.n.) routes (13). LT-IIb and LT-IIIbT13I each significantly enhanced the onset and magnitude of RiVax-specific serum IgG levels and protective immunity in mice, demonstrating the general compatibility of RiVax with the HLT adjuvants. We now report a systematic comparison of the adjuvant activities of LT-IIa, LT-IIb, LT-IIIbT13I, and LT-Iic when administered to mice by the i.d. route in conjunction with RiVax and RVEc. The results of the study indicate that while all four HLTs tested were effective adjuvants, LT-IIa proved the most promising because of its effectiveness even at subinflammatory doses.

MATERIALS AND METHODS

Chemicals, reagents, and cell lines. Ricin was purchased from Vector Laboratories (Burlingame, CA) and dialyzed against phosphate-buffered saline (PBS) at 4°C in 10,000-molecular-weight (MW)-cutoff Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, IL) prior to use in cytotoxicity and mouse studies. RiVax (lot 190-100L-GLP-FF-090105) in 144 mM NaCl, 10 mM histidine, pH 6.0, in 50% glycerol was obtained from Soligenix, Inc. RVEc (lot 011314a) in 20 mM sodium succinate, 100 mM NaCl, 0.12% Tween 20 at pH 6.5 was obtained from Leonard Smith and Ralph Tammariello at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID; Fort Detrick, MD). Recombinant His-tagged LT-IIa, LT-IIb, LT-IIIbT13I, and LT-Iic were purified from E. coli (10). Alhydrogel aluminum adjuvant (batch 4772) was obtained from Brenntag (Mülheim an der Ruhr, Germany). Vero cells were purchased from the American Type Culture Collection (Manassas, VA). Cell lines were maintained in a humidified incubator at 37°C with 5% CO2. Unless noted specifically, all other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Immunization protocols. Female BALB/c mice 7 to 12 weeks of age were obtained from Taconic Laboratories (Hudson, NY) or Harlan Laboratories (Madison, WI). Animals were housed under conventional, specific-pathogen-free conditions and were treated in compliance with the approval of the local Institutional Animal Care and Use Committee (IACUC) guidelines at the Wadsworth Center and the University at Buffalo. Immunization studies were conducted with 5 to 10 mice per group, as specified in Results and figure legends. RiVax or RVEc was mixed with LT-II adjuvants or adsorbed to Alhydrogel (0.85 mg/ml) for 2 h just prior to immunization. Intraderal immunizations were performed via the intraderal (i.d.) route with each injection containing a 30- to 40-μg dose of ricin (0.2 μg/mouse). Survival was monitored over a 7-day period. Hypoglycemia was used as a surrogate marker of ricin intoxication (14). Mice were euthanized when they became overtly moribund and/or blood glucose levels fell below 25 mg/dl.

Analysis of skin inflammation. Mice were immunized by the i.d. route with RiVax and LT-II adjuvants and were observed daily for reactivity at the site of immunization. For gross morphological analysis, edema was measured using digital calipers. Edema was determined by taking two orthogonal measurements (M1 and M2, M2 > M1) of the edema diameter and then multiplied by an estimated depth, which was arbitrarily set at M1 because of the difficulty in accurately measuring the depth of swelling, especially at early time points. Hence, edema volume was calculated as M1 × M2 × M2, and values were reported as cubic millimeters as done previously (9).

ELISA. Nunc 96-well plates were coated overnight at 4°C with ricin (1 μg/ml) and then blocked for 2 h with 2% goat serum in 0.1% PBS-Tween (PBST). Twofold serial dilutions of serum, starting from 1:100, were then applied in duplicate for 1 h, washed, and detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG for 1 h. After another wash, the reaction was developed with SureBlue tetramethylbenzidine (TMB) (KPL, Gaithersburg, MD), and quenched with 1 M phosphoric acid before being scanned at 450 nm on a VersaMax microtiter plate spectrophotometer (Molecular Devices, Sunnydale, CA). The endpoint titer, determined by direct ricin toxin enzyme-linked immunosorbent assay (ELISA), was defined as the lowest dilution whose absorbance (450 nm) was >3 times background. Seroconversion was defined as an endpoint titer of ≥1:100. Geometric mean titers (GMTs) were calculated from the endpoint titers. The endpoint titer for any mouse that had not seroconverted was set to 1 for GMT calculations.

Ricin toxin neutralization assays. Vero cells were trypsinized, adjusted to ~5 × 105 cells/ml, seeded (100 μl/well) into white-bottom 96-well plates (Corning Life Sciences, Corning, NY), and allowed to adhere overnight. The cells were treated with ricin (10 ng/ml), ricin-serum mixtures, or culture medium (negative control) for 2 h at 37°C. Cells were then washed to remove noninternalized ricin or ricin–serum mixtures and incubated for 48 h at 37°C. Cell viability was assessed using CellTiter-Glo (Promega, Madison, WI). All treatments were performed in triplicate, and 100% viability was defined as the average value obtained from wells in which cells were treated with culture medium only. The percent viability was defined as the percentage of Vero cells that were protected from the effects of ricin (10 ng/ml) at a given serum dilution (1:50 or 1:100).

Statistical analysis and software. Statistical analysis was carried out with GraphPad Prism 5 (GraphPad Software, San Diego, CA). Endpoint titers were log transformed prior to statistical analysis. Endpoint and neutralizing titers were compared using 1-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. Edema measurements were compared with a 1-way ANOVA, followed by the Newman-Keuls multiple comparison test. Survival data were tested using the log rank Mantel-Cox test. In all cases, the significance threshold was set at P < 0.05.

RESULTS

LT-II adjuvants stimulate ricin-specific serum IgG and toxin-neutralizing titers when coadministered with RiVax or RVEc. We first compared the adjuvant activities of the four different LT-II enterotoxins, LT-IIa, LT-IIb, LT-IIIbT13I, and LT-Iic, each combined with high (5-μg) or low (0.5-μg) doses of RiVax and administered to BALB/c mice by the i.d. route. Mice were primed on day 0, boosted on days 10 and 20, and then challenged with 10 LD50's of ricin toxin on day 34. Sera were collected from the mice on days 17 and 27 and examined for toxin-specific serum IgG levels and TNA.

Analysis of sera collected on day 17 from animals that received high- or low-dose RiVax without adjuvant indicated seroconversion rates of ~60% and extremely low GMTs (30 to 47) (Table 1; Fig. 1). In contrast, groups of mice that received high-dose RiVax plus one of the LT-II adjuvants had a seroconversion rate of 100% and GMTs ranging from 5,400 (in the case of LT-IIIbT13I) to 47,600 (in the case of LT-IIa) (Table 1; Fig. 1). Mice that received low-
TABLE 1 Adjuvant activities associated with LT-II toxins when coadministered with RiVax and RVEc

| LT-II enterotoxin | RiVax (5 μg) | RiVax (0.5 μg) | RVEc (0.5 μg) |
|-------------------|--------------|----------------|---------------|
|                    | GMT (day)    | GMT (day)      | GMT (day)     |
|                    | 17 27 | % Viab | Survival | 17 27 | % Viab | Survival | 17 27 | % Viab | Survival |
| LT-IIa            | 47,771 | 95,543 | 45a | 1/10 | 12,800 | 27,437 | 25a | 1/10 | 10,397 | 31,517 | 13a | 1/10 |
| LT-Ib             | 11,143 | 38,802 | 17 | 1/10 | 4,525 | 33,779 | 32a | 1/10 | 7,352 | 29,407 | 39a | 1/10 |
| LT-IIb (T13I)     | 5,486 | 44,572 | 20 | 1/10 | 3,430 | 19,401 | 18 | 1/10 | 6,000 | 19,401 | 13 | 1/10 |
| LT-Iic            | 15,759 | 5,340 | 4 | 1/10 | 5,486 | 23,407 | 20 | 1/10 | 2,263 | 6,340 | 14 | 1/10 |

a. Ricin subunit antigen and dose used in the experiment.
b. Geometric mean endpoint titer.
c. Mean cell viability achieved by a 1:50 dilution of serum collected on day 27 for groups receiving 0.5 μg RiVax or RVEc or 1:100 for serum collected on day 27 from mice that received 5 μg RiVax.
d. Number of mice to survive a 10 LD50 ricin challenge on day 34 out of 10 total mice. Sham-immunized mice (not shown) all succumbed to ricin challenge, as shown in the accompanying figures.
e. Adjuvant in a given experiment at a dose of 1 μg.
f. P < 0.05 vs RiVax or RVEc without adjuvant.

dose RiVax plus one of the LT-II adjuvants also demonstrated 100% seroconversion, although GMTs were considerably lower (range, 1,200 to 12,800). By day 27, all mice that received high- or low-dose RiVax even without adjuvant had seroconverted. In the high-dose RiVax group, the GMT increased ~360-fold (GMT of 16,890) compared to day 17, while the low-dose group GMT increased 100-fold (GMT of 3,940). On day 27, the GMTs of mice that received high- or low-dose RiVax plus any one of the LT-II adjuvants were significantly higher than GMTs of mice that received RiVax only, underscoring that fact that the LT-II adjuvants not only accelerated the onset of detectable ricin-specific serum IgG titers but ultimately increased the absolute magnitude of the antigen-specific antibody responses. All animals that were vaccinated with RiVax (irrespective of the presence or absence of adjuvant) survived ricin toxin challenge administered on day 34 (Table 1).

Among the four adjuvants tested, LT-Iia tended to elicit the highest ricin-specific endpoint titers on day 17. For example, in conjunction with high-dose RiVax, LT-Iia elicited a GMT after a single prime-boost regimen that was ~3-fold higher than the response elicited by prime and two boosts with RiVax alone (Table 1). Furthermore, a prime-boost regimen of low-dose RiVax with LT-Iia elicited ricin-specific GMTs that were roughly equivalent to those elicited by prime and two boosts with high-dose RiVax (Table 1).

To assess the “quality” of the serum antibody responses elicited by RiVax with LT-II adjuvants, sera were evaluated for TNA. We and others have recently reported that toxin-neutralizing titers generally lag behind total ricin-specific IgG levels by 1 to 2 weeks, a phenomenon that we note in Discussion (15, 16). It was, therefore, not surprising that serum samples from day 17 were devoid of detectable in vitro TNA (data not shown). However, by day 27, TNAs were evident in all groups, with the lowest values associated with mice that received RiVax without adjuvant (Table 1; Fig. 2). The highest TNA values were associated with mice that received RiVax plus LT-Iia or LT-Iib (Table 1; Fig. 2).

We next evaluated the degree to which the individual LT-II enterotoxins enhanced the immune response to RVEc, a truncated RTA-based subunit vaccine antigen (12, 17). Low-dose RVEc (0.5 μg) was mixed with each of the four different LT-II enterotoxins and administered to groups of BALB/c mice by the i.d. route. Analysis of sera collected on days 17 and 27 indicated that ricin-specific GMTs were significantly higher in LT-II-treated groups of mice than in mice that received only RVEc (Table 1; Fig. 1). The highest TNA values were again associated with the sera from animals immunized with LT-Iia or LT-Iib (Table 1; Fig. 2). Collectively, these data demonstrate that all four LT-II enterotoxins have adjuvant activity when combined with RiVax or RVEc, with the most potent being LT-Iia and LT-Iib.

Local inflammation associated with LT-II adjuvants. We determined the acute inflammatory response associated with each of the LT-II adjuvants when administered in conjunction with RiVax to mice by the i.d. route. We previously reported that LT-Iib is relatively inflammatory, as evidenced by significant and persistent edema at the site of injection that was characterized by extensive fluid accumulation and cellular infiltration (13). LT-Iib (T13I), on the other hand, was deemed largely nonreactogenic. As described in Materials and Methods, each of the LT-II enterotoxins (1 μg) was mixed with RiVax (5 μg) and delivered to mice by the i.d. route, after which edema was scored daily for up to 10 days. We found that LT-Iib was the most inflammatory, with a maximal edema volume of about 2,500 mm³ 3 days after administration, while LT-Iib (T13I) was the least inflammatory (Fig. 3A). LT-Iia and LT-Iic elicited intermediate levels of inflammation, at least when administered at 1 μg.

Adjuvant activity of LT-Iia at nominally inflammatory levels. Although LT-Iia was the most effective among the four LT-II enterotoxins at serving as an adjuvant for RiVax and RVEc, it demonstrated intermediate levels of inflammation when administered at the standard dose of 1 μg per injection. We therefore sought to examine whether subinflammatory or minimally inflammatory amounts of LT-Iia still retained significant adjuvant activity. We first performed a dose-response study in which mice received 3-fold dilutions of LT-Iia (range, 1.0 to 0.03 μg) mixed with RiVax (5 μg) by the i.d. route. Edema was measured over a 10-day period. As shown in Fig. 3B, inflammation associated with LT-Iia was indeed dose dependent. The smallest amount of LT-Iia tested, 0.03 μg, showed no evidence of edema compared to RiVax except for a slight increase on day 2. For that reason, we considered 0.03 μg LT-Iia to be nominally inflammatory.

To determine if the low doses of LT-Iia are sufficient to enhance antigen-specific immune responses, different amounts of LT-Iia (range, 1.0 to 0.03 μg) were combined with RiVax and...
RVEc and administered to mice by the i.d. route. Analysis of sera collected 7 days after a prime and one (Fig. 4A) or two (Fig. 4B) boosts indicated that even the smallest amount of LT-IIa tested (0.03 μg) gave rise to ricin-specific serum IgG GMTs that were significantly higher than those from mice that received RiVax or RVEc without adjuvant (Table 2; Fig. 4A and B). Low-dose LT-IIa (0.03 μg) was also sufficient to elicit ricin toxin-neutralizing antibodies to levels significantly above those elicited by antigen alone and roughly half of that elicited by high-dose LT-IIa (1.0 μg) (Table 2; Fig. 4C). Finally, mice immunized with a prime and a single boost of low-dose LT-IIa (0.03 μg) in conjunction with RVEc were fully protected against 10-LD₅₀ ricin challenge (Fig. 4D).

Adjuvant activity of LT-IIa compared to Alhydrogel. Although aluminum salt is not an adjuvant normally administered via the i.d. route, it does constitute the standard against which new adjuvants are generally compared (1). For this reason, we chose to perform a comparative vaccination study between Alhydrogel.
and LT-IIa (0.03 mg/ml) in conjunction with high- and low-dose RV
Ec. Groups of mice were immunized by the i.d. route on days 0 and 10. On day 17, each group was divided into two subgroups: one subgroup was challenged with ricin on day 22, while the other was monitored over a 6-month period for the onset of ricin-specific GMT and TNA before being challenged with ricin on day 203. LT-IIa and Alhydrogel were virtually identical with respect to their abilities to adjuvant RV
Ec, as evidenced by nominal differences in GMT, TNA, and survival on days 22 and 203 (Fig. 5; also see Tables S1 and S2 in the supplemental material).

Taken together, these results demonstrated that LT-IIa was as effective as Alhydrogel in serving as an adjuvant for RV
Ec when delivered i.d.

In a separate but related study, we investigated the potential of LT-IIa to work in concert with Alhydrogel. In this experiment, groups of mice received i.d. injections of low-dose RVEc plus LT-
IIa, Alhydrogel, or a combination of the two on days 0 and 10. We also evaluated LT-IIa in combination with LT-IIb T13I using the same regimen. Analysis of serum antibody responses on day 17 demonstrated that all three adjuvants administered individually enhanced ricin toxin-specific serum IgG levels over those observed in RVEc-vaccinated mice but that no additional benefit was observed by combining adjuvants (see Fig. S1 in the supplemental material). Thus, the adjuvant effects of LT-IIa are not additive to those observed with aluminum salts or LT-IIbT13I.

**DISCUSSION**

The type II heat-labile enterotoxins constitute a promising set of adjuvants that have been shown to enhance systemic and mucosal humoral and cellular responses to model antigens and several pathogen-associated antigens (7). In this study, we compared the four best-studied type II HLTs, namely, LT-IIa, LT-IIb, LT-IIbT13I, and LT-IIc, for their abilities to serve as adjuvants for two different clinically relevant protein subunit antigens, RiVax and RVEc, in a mouse model. While all four enterotoxins tested had significant adjuvant activity and are worthy of further investigation, LT-IIa was of particular interest in that extremely low doses of LT-IIa proved sufficient to enhance the onset and magnitude of ricin-specific serum IgG levels with only nominal local inflammatory responses. The fact that the adjuvant and inflammatory ac-

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**TABLE 2** Effect of LT-IIa on ricin-specific GMT and TNA when combined with RiVax or RVEc

| LT-IIa (μg) | GMT  | Viab²  | GMT  | Viab²  | GMT  | Viab²  |
|------------|------|--------|------|--------|------|--------|
|            | Day 17 |       | Day 27 |       |       |       |
| RVEc (2 or 3×) on day 17* |   |       |   |        |   |        |
| 1          | 18,102 | 1      | 33,779 | 4      | 72,408 | 90     |
| 0.3        | 18,102 | 1      | 15,759 | 2      | 41,587 | 65     |
| 0.1        | 11,404 | 0      | 5,198  | 1      | 31,517 | 59     |
| 0.03       | 11,404 | 2      | 7,352  | 2      | 54,875 | 54     |
| 0          | 1,008  | 0      | 159    | 1      | 16,890 | 9      |

* Antigen and number of immunizations. Both antigens were administered at 5 μg.

² Mean Vero cell viability with sera diluted 1:50, as described in Materials and Methods.
Activities can be uncoupled increases the likelihood that LT-IIa will be amenable for use in humans (1).

The immunomodulatory properties of LT-IIa are distinct from those elicited by CT, LT-I, or the other type II HLTs. LT-IIa, originally identified from a strain of *E. coli* isolated from water buffalo, has a unique ganglioside binding profile summarized as binding avidly to GD1b and secondarily to GD1a, GM1, and others (18, 19). When administered intranasally to mice, LT-IIa induce a balanced Th1 and Th2 cytokine profile with subsequent enhancement of IgG1, IgG2a, IgG2b, and mucosal IgA responses (20).

![FIG 5](https://cvi.asm.org/)

**FIG 5** Comparison between LT-IIa and Alhydrogel as adjuvants in conjunction with RVEc. Groups of BALB/c mice (*n* = 10 per group) were immunized by the i.d. route on days 0 and 10 with low- or high-dose RVEc adsorbed to Alhydrogel (Alum) or combined with LT-IIa (0.03 μg), as described in Materials and Methods. On day 22, each group of animals was divided into two groups of five. Groups of mice were challenged with ricin on day 22 or 203. (A and B) Ricin-specific GMTs in sera collected on day 17 (*n* = 10) (A) or 199 (*n* = 5) (B). (C to F) Survival curves for groups of animals (*n* = 5/group) that received high- (C and D) or low-dose (E and F) RVEc and were challenged on day 22 (C and E) or 203 (D and F). Symbols: circles, LT-IIa; squares, Alhydrogel; triangles, RVEc; crosses, control mice. When two or more lines on the graph overlapped because of identical survival patterns (e.g., 100% survival), the “nudge” option was applied in GraphPad to eliminate overlap and make each line visible. *, *P* < 0.05.
cific CD8⁺ T cells (21). Mathias-Santos and colleagues tested LT-IIa’s adjuvant activity at only a single dose (0.5 µg). It would be interesting to examine antigen-specific CD8⁺ T cell responses with low-dose LT-IIa in light of our finding that LT-IIa retains adjuvant activity (e.g., induction of serum IgG) in the low-nanogram level.

It has been reported that ADP-ribosylating toxins like CT and LT, when administered as adjuvants to rodents and humans by the transcutaneous route, stimulate the production of antigen-specific mucosal antibody responses (22–24). By extension, one might expect that LT-II enterotoxins administered i.d. might also induce antigen-specific secretory IgA (sIgA) antibody responses. Eliciting mucosal immune responses to ricin toxin is obviously beneficial, considering its toxicity following inhalation or ingestion (25–27). In the current study, we did assess, using protocols previously established in our laboratory (28), ricin-specific IgA antibodies in fecal pellets from mice immunized with RiVax and the four different LT-IIa adjuvants. However, in no instance did we detect levels of IgA above background (data not shown), suggesting that the four LT-II adjuvants tested here do not communicate with the gastrointestinal mucosal immune system following i.d. delivery. We did not extend our studies to include lung or bronchial alveolar lavage.

Results presented in the current study extend a previous report from our laboratory indicating that RiVax and RV Ec are effectively indistinguishable with respect to their ability to elicit ricin-specific serum IgG titers, toxin-neutralizing activity, and protective immunity to ricin (12). In the previous study, RiVax and RV Ec were adsorbed onto Alhydrogel and then administered to mice by subcutaneous (s.c.) injection. We have now demonstrated that RiVax and RV Ec are indeed identical to each other even when administered to mice with type II HLT adjuvants rather than aluminum salts and by i.d. immunization rather than s.c. immunization.

Another point worth noting is that the onset of TNA following immunization with RV Ec, even with the addition of LT-IIa, generally lagged well behind peak or near-peak ricin-specific GMTs. This lag in TNA relative to total toxin-specific antibody levels has been noted in previous studies conducted in mice (16) and, very recently, in rhesus macaques (15). In the former study, groups of mice received RiVax, a modified anthrax protective antigen (PA), or a combination of the two. By day 20 following a prime-boost regimen, we noted that 19/20 PA-immunized mice had anthrax lethal toxin-neutralizing antibodies, whereas 1/20 RiVax-immunized mice had ricin-specific TNA. We speculated in that study that it may be intrinsically more difficult to neutralize ricin than other toxins because of a limited number of neutralizing B cell epitopes on RTA (29, 30). As a result, potent ricin-neutralizing antibodies may not arise until a certain threshold of specificity and/or avidity is achieved that is above that for other protein toxins. We can conclude from our current study that the observed lag in TNA following RV Ec vaccination is not limited to the use of aluminum salts adjuvant as we previously had suggested, since the same effects were observed with LT-II enterotoxins.

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