Differential Binding of *Escherichia coli* Enterotoxins LT-IIa and LT-IIb and of Cholera Toxin Elicits Differences in Apoptosis, Proliferation, and Activation of Lymphoid Cells

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Cholera toxin (CT), LT-IIa, and LT-IIb are potent adjuvants which induce distinct T-helper (Th)-cell cytokine profiles and immunoglobulin G (IgG) subclass and IgA antibody responses. To determine if the distinct immune regulatory effects observed for LT-IIa, LT-IIb, and CT are elicited by binding of the enterotoxins to their cognate ganglioside receptors, the lineages of lymphoid cells that interact with the three enterotoxins and their effects on various lymphocyte responses in vitro were evaluated. Binding patterns of LT-IIa, LT-IIb, and CT to several lymphoid cell populations were distinctive for each enterotoxin. LT-IIa and CT, but not LT-IIb, induced apoptosis in CD8+ T cells. LT-IIa(T34I), a mutant with no detectable binding to gangliosides, did not induce apoptosis. Blockade of GM1 on the surface of CD8+ T cells by LT-IIa(T14I), a mutant that binds only to GM1, but does not induce apoptosis, did not inhibit induction of apoptosis by LT-IIa. Mitogen-induced proliferation of CD8+ T cells was abrogated by treatment with CT, while resting CD8+ T cells which were sensitive to LT-IIa-induced apoptosis became more resistant to apoptosis after mitogen activation. Exposure to CT, but not to LT-IIa or LT-IIb, inhibited mitogen-driven CD4+ T-cell proliferation and expression of CD25 and CD69. In mitogen-stimulated B cells, CT, but not LT-IIa or LT-IIb, enhanced expression levels of CD86, while only CT induced B-cell differentiation into plasma cells. Thus, LT-IIa, LT-IIb, and CT exhibit distinguishable immunomodulatory properties which are likely dependent upon their capacities to recognize different ganglioside receptors on lymphocytes.

The heat-labile enterotoxins (HLT) of *Vibrio cholerae* and *Escherichia coli* belong to a family of structurally related bacterial toxins that induce diarrheal symptoms in humans and animals when expressed by the bacterial cells in the lumen of the gut. HLT are oligomeric proteins composed of a single A polypeptide which is noncovalently bound to a pentameric array of B polypeptides. The toxic effects of HLT are determined by the binding specificity of the B pentamers for cell surface receptors and by the ADP ribosylating properties of the A subunit (17, 29, 48).

Two types of HLT have been distinguished on the basis of distinct immune reactivities (29, 44): the type I HLT include *V. cholerae* toxin (CT) and the *E. coli* enterotoxin LT-I (29, 30); the type II HLT include LT-IIa and LT-IIb, two antigenically related enterotoxins produced by certain strains of *E. coli* (1, 19, 21, 22). A comparison of the predicted amino acid sequences of the type I and type II HLT reveals both conservation and variability (29, 43–45). The A polypeptides of type I HLT and type II HLT are highly homologous, which is reflected in their closely similar ADP ribosylating activities. In contrast, the B polypeptides of the two classes of HLT exhibit significant divergence in amino acid sequence, which imparts upon the molecules the range of receptor binding specificities observed for the enterotoxins.

The functional receptors for type I and type II HLT are gangliosides, a family of structurally complex glycolipids which reside in the plasma membrane of eukaryotic cells. Expression of different gangliosides varies widely at the cell, tissue, and organ levels and between mammalian species (40). While the physiological roles of gangliosides are not well established, these molecules have been shown to influence events that lead to cellular activation, proliferation, and differentiation in various cellular types (9, 24, 41, 42, 49, 54). In vitro binding assays have shown that the members of the type I and type II HLT exhibit differences in their relative binding affinities for various gangliosides. CT and LT-I bind with high affinity to ganglioside GM1. A more divergent pattern of ganglioside binding is observed for LT-IIa, which binds most avidly to ganglioside GD1a and with less avidity to gangliosides GD1b and GM1. LT-IIb binds with high affinity only to GD1a (14).

Despite their inherent enterotoxicities, LT-IIa, LT-IIb, and CT have been successfully employed as adjuvants in experimental animals to enhance mucosal and systemic antibody responses to coadministered antigens (Ags) (3, 4, 11, 35, 37–39). While LT-IIa, LT-IIb, and CT have potent adjuvant activities, the adjuvant properties of the three enterotoxins are distinctive (35, 37–39, 51, 52). Using a mouse model and a streptococcal Ag, CT was shown to induce a predominant Th2 response, which is associated with increased production of interleukin-4 (IL-4), IL-5, and IL-10, and subsequent elevated levels of Ag-specific immunoglobulin G1 (IgG1) antibodies.
Using the same model system, it was shown that LT-IIa and LT-IIb elicit a more balanced Th1 and Th2 cytokine profile and a more unbiased Ag-specific IgG subclass antibody response (37, 38).

It is clear from a variety of investigations that the immune regulatory activities of CT and LT-I depend critically upon the binding affinity of the enterotoxin for GM₁ (20, 42). The effects of ganglioside binding on the immune regulatory activities of LT-IIa and LT-IIb, however, have not been rigorously evaluated. To investigate the hypothesis that the distinct immune regulatory effects elicited by LT-IIa, LT-IIb, and CT are the result of binding of these enterotoxins to different ganglioside receptors, the pattern of binding of those three enterotoxins to lymphoid cell populations was evaluated. Lymphoid cells were analyzed for costimulatory molecule expression, apoptosis, and mitogen-stimulated responses of B and T lymphocytes to determine if binding of the enterotoxins influenced cellular and molecular activities which are associated with immunoregulation. Results of the investigation revealed that LT-IIa, LT-IIb, and CT have differing capacities to influence immune responses and that the ganglioside binding affinities of the enterotoxins likely regulate those immunomodulatory activities.

MATERIALS AND METHODS

Expression and purification of recombinant enterotoxins. Recombinant entero toxins and their respective B pentamer were purified from cellular extracts by a combination of nickel affinity and gel filtration chromatography, as previously reported (23).

Mice and cells. To produce single-cell suspensions, spleens, aseptically harvested from female BALB/c mice of 8 to 12 weeks of age, were pressed through a cell strainer (Becton-Dickinson, San Jose, CA) into phosphate-buffered saline (PBS; Gibco BRL, Gaithersburg, MD). Mononuclear cells were isolated from the cell mixture by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation. Cells were washed two times with PBS to remove the Ficoll-Hypaque. The number of viable cells in the enriched population, determined by use of trypan blue (Invitrogen, Carlsbad, CA) dye exclusion, was routinely ≥90% of total cell counts.

Enterotoxin binding assay. Splenic cell populations (2 × 10⁶ cells) from naive mice were treated in vitro with 1 µg of LT-IIa, LT-IIa(T141I), LT-IIa(T34I), LT-IIb, or CT holotoxin. After incubation on ice for 10 min, cells were washed with buffer (PBS, 3% bovine serum albumin, 0.05% NaN₃) and incubated on ice for 10 min with a pretitrated concentration of rabbit anti-LT-IIa, anti-LT-IIb, or anti-CT antiserum produced in this laboratory. After washing to remove unbound antibodies, cells were treated with 0.5 µg/ml of phycoerythrin-conjugated goat anti-rabbit IgG (Caltag Laboratories, Burlingame, CA) and with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against the following lymphoid cell surface markers: CD3, CD4, CD8, B220, CD11c, and CD11b (PharMingen, San Diego, CA). After 10 min, cells were washed as described above, incubated with 1 µg/ml of propidium iodide (PI; Sigma Biologicals, St. Louis, MO), and analyzed by fluorescence-activated cell sorting (FACS). CD16/CD32 (PharMingen, San Diego, CA) antibodies were used for blocking Fc receptor as indicated by the manufacturer. Isotype-matched fluorochrome-labeled antibodies and specific antienterotoxin rabbit serum were used as controls for binding.

Purification of CD8⁺ cells. Single-cell suspensions from spleens were incubated with anti-CD8/32 followed by incubation for 20 min at 6°C with CD8α microbeads (Miltenyi Biotec, Auburn, CA). CD8⁺ cells were magnetically selected by two successive rounds of enrichment using LS positive selection columns (Miltenyi Biotec, Auburn, CA). The positive fraction, analyzed by FACS, routinely contained ≥95% of CD8⁺ cells.

Lymphocyte activation and proliferation assays. Splenic cells were labeled with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) (36), suspended in complete RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) containing 10% fetal calf serum, and then added to 24-well culture plates (2 × 10⁶ cells/ml/well) containing 2 µg/ml of concanavalin A (ConA; Sigma, St. Louis, MO) or 2 µg/ml of E. coli lipopolysaccharide (LPS; Calbiochem, San Diego, CA). Cell cultures were treated with either LT-IIa, LT-IIb, or CT at a concentration of 1 µg/ml. Cultures, incubated at 37°C in humidified air plus 5% CO₂ for 3 to 4 days, were stained for CD4, CD6, B220, CD40, CD80, CD86, or CD138 (PharMingen, San Diego, CA) and PI, as described above. The amount of cell proliferation in the cell populations was quantified by monitoring the sequential loss of fluorescence intensity of the cells using FACS. CFSE irreversibly couples to intracellular proteins, which are equally distributed between the two daughter cells after cell division (26, 36).

Cell death assays. Splenic mononuclear cells (2 × 10⁶ cells/ml) were incubated in complete RPMI containing 10% fetal calf serum at 37°C in humidified air plus 5% CO₂. Cultures were treated, as appropriate, with 1 µg/ml of LT-IIa, LT-IIb, or CT for 3 days. At harvesting, cells were washed two times with PBS and stained for CD3, CD8, and PI. In addition, isolated CD8⁺ cells (2 × 10⁶) for FACS analysis were incubated for 24 h with 1 µg/ml of wild-type (wt) LT-IIa or LT-IIa(T34I) and subsequently stained with Annexin V-FITC plus PI (Annexin V-FITC apoptosis detection kit II; PharMingen, San Diego, CA).

FACS. Sample acquisition and analysis were performed using a FACScalibur flow cytometer (Beckton-Dickinson) and CellQuest software (Beckton-Dickinson). Experiments were repeated at least three times. No less than 10⁴ events were acquired for each experiment. PI staining was used in all instances to exclude nonviable cells from the analyses.

RESULTS

Binding of enterotoxins to lymphoid cells. LT-IIa, LT-IIb, and CT have affinities for different gangliosides, a class of molecules which have been shown to have important bioactive functions in cell membranes. Results from recent investigations provided the impetus to investigate the hypothesis that the immune regulatory activities of LT-IIa, LT-IIb, and CT were governed both by the lymphoid cell types to which they bind and by the receptor binding specificity of each enterotoxin (37–39). As an initial approach to evaluate that hypothesis, the types and frequencies of lymphoid cells which interact with LT-IIa, LT-IIb, and CT were determined. Single-cell suspensions from spleens which had been incubated with each of the enterotoxins were analyzed by FACS for the presence of enterotoxin binding cells (Fig. 1). Analysis was limited to those cells expressing CD3 (total T cells), B220 (B cells), CD11b (macrophages), or CD11c (dendritic cells). FACS analysis showed that all cell populations were bound by CT, indicating that GM₁ is expressed on all splenic lymphoid cells. A substantial fraction of B220⁺ cells (~70%) was positive for binding LT-IIb, while only ~30% of B220⁺ cells were bound by LT-IIa. LT-IIb and LT-IIa bound to ~30% and ~65% of CD3⁺ cells, respectively. To extend the analysis, T-cell populations were further differentiated to CD4⁺ and CD8⁺ populations. Essentially ~20% of CD4⁺ cells were positive for binding LT-IIb and ~55% of CD4⁺ cells were positive for binding LT-IIa. LT-IIb and LT-IIa bound to ~60% and ~90% of CD8⁺ cells, respectively. Additional FACS analysis demonstrated that LT-IIa and LT-IIb also bound to the majority of CD11b⁺ and to CD11c⁺ cells (~90%). Taken together, these results demonstrated that GM₁, GM₂, and GM₃ are likely expressed on certain subpopulations of immune cells. The distinct binding patterns of LT-IIa, LT-IIb, and CT to lymphoid cells supported the hypothesis that these enterotoxins recognize different ganglioside receptors located on the cell surface of various lymphoid cell populations. Additional experiments were performed to determine if binding of the enterotoxins to the lymphoid cells elicited cellular events which are commonly associated with immunomodulation and immune responsiveness.
Apoptosis induction and proliferation of CD8<sup>+</sup> cells. Signaling through different ganglioside receptors likely elicits distinct cellular responses in lymphoid cells. For example, cross-linking of GM<sub>1</sub> by CT or LT-I is known to induce apoptosis of CD8<sup>+</sup> T cells (2, 42, 49, 53). Thus, the influence of LT-IIa or LT-IIb on apoptosis induction and proliferation of CD8<sup>+</sup> lymphocytes was evaluated. Splenic single-cell suspensions were cultured for 3 days in the presence or absence of LT-IIa, LT-IIb, or CT. On harvest, cells were stained with CD3 and CD8, and the frequencies of viable cells coexpressing these two lymphoid cell markers were determined by FACS (Fig. 2). CD8<sup>+</sup> cells were depleted in cultures treated with LT-IIa or CT, whereas untreated control cultures and cultures treated with LT-IIb exhibited no detectable changes in CD8<sup>+</sup> populations. The decrease in the proportions of CD8<sup>+</sup> cells in cultures treated with either LT-IIa or CT was not due to selective proliferation of other cell types, since no proliferation, as assessed by CFSE cell division tracking, was observed in the enterotoxin-treated cultures (data not shown). In addition, the decrease in CD8<sup>+</sup> cells was not a result of down-regulation of CD8 Ag expression on the cultured splenic cells. Depletion of cell numbers was also evident in cultures of purified CD8<sup>+</sup> cells which had been treated with LT-IIa (see Fig. 4B, below) or CT (data not shown).

Given that LT-IIa binds GD<sub>1a</sub>, GD<sub>1b</sub>, and GM<sub>1</sub> and that LT-IIb, which binds only to GD<sub>1a</sub>, did not induce death of

![Figure 1](http://iai.asm.org/) Binding of LT-IIa, LT-IIb, and CT to splenic mononuclear cells. Splenic cell suspensions were incubated with enterotoxin and counterstained for CD3, CD4, CD8, B220, CD11b, and CD11c. Histograms are the representative fluorescence profiles gated to demonstrate binding of enterotoxins to cells expressing CD3, CD4, CD8, B220, CD11b, or CD11c.

![Figure 2](http://iai.asm.org/) LT-IIa and CT, but not LT-IIb, induce depletion of CD8<sup>+</sup> cells. Splenic cell suspensions were cultured for 3 days in the absence or presence of 1 µg/ml of LT-IIa, LT-IIb, or CT. Cells stained for CD3 and CD8 were analyzed by FACS for frequencies of viable cells expressing the two lymphoid cell markers.
CD8⁺ cells, it was hypothesized by subtractive analysis that LT-IIa likely depletes CD8⁺ cells via a mechanism involving interaction with either GD₁₉₉ or GM₁, or with both gangliosides. To differentiate between these two possibilities, GM₁ on the surface of lymphoid cells was blocked by pretreatment of cells with saturating concentrations of the single point substitution mutant LT-IIa(T14I). LT-IIa(T14I) binds to GM₁ (5) and to CD8⁺ cells (Fig. 3A) but has no detectable binding affinity for GD₁₉₉ or GD₁₁₀ (5). Notably, LT-IIa(T14I) did not elicit depletion of CD8⁺ T cells in our assays and, as such, was a neutral participant in these depletion experiments (Fig. 3B). After blocking, cells were incubated for 3 days with wt LT-IIa, after which the frequency of viable CD8⁺ T cells was determined by FACS (Fig. 3C). Blockade of GM₁ with LT-IIa(T14I) did not inhibit LT-IIa-associated depletion of CD8⁺ cells. These data are consistent with a model in which LT-IIa triggers death of CD8⁺ cells by signaling pathways associated with binding to GD₁₁₀, but not involving binding to GM₁.

Death of CD8⁺ cells by LT-IIa treatment could have occurred by one of two mechanisms: necrosis or programmed cell death (apoptosis). To determine which of these mechanisms mediated LT-IIa-associated CD8⁺ cell depletion, purified CD8⁺ cells were cultured for 24 h with either wt LT-IIa or LT-IIa(T34I). LT-IIa(T34I) lacks detectable binding affinity for GD₁₉₉, GD₁₁₀, and GM₁ (5) and does not bind to lymphocytes (Fig. 4A). Cells were stained with Annexin V and PI to differentiate between early apoptotic and necrotic cells. Early apoptotic cells bind Annexin V and can be distinguished from late apoptotic and necrotic cells by their ability to exclude PI (12, 47). In comparison to cells treated with LT-IIa(T34I), a higher proportion of early and late apoptotic cells were observed in cultures of CD8⁺ cells treated with LT-IIa (Fig. 4B).

In addition, in cultures treated with wt LT-IIa, but not in those treated with LT-IIa(T34I), a progressive decrease in the light-scattering properties of cells was observed over time. Given that a decrease in the forward light scatter of cells has been reported for other apoptosis systems (7), this result suggested a progression of cells from an early to an advanced stage of apoptosis. Taken together, these results strongly indicated that LT-IIa-associated depletion of CD8⁺ cells was likely an apoptotic event and that GD₁₉₉ occupancy by the enterotoxin was essential for eliciting the apoptotic effect. Furthermore, since apoptosis could be induced in purified preparations of CD8⁺ cells, these results indicate that apoptosis induction by LT-IIa was not due to the actions exerted by other cell types or cytokines that otherwise may be present in unfractonated cell cultures.

As susceptibility to apoptosis often depends upon cell cycle stage (31, 33, 46), the effect of activation-proliferation on the propensity of CD8⁺ cells to undergo enterotoxin-induced cell death was determined. Herein, CFSE-labeled splenocytes were stimulated with ConA for 4 days in the presence or absence of LT-IIa, LT-IIb, or CT, and CD8⁺ cells in the splenic population were then analyzed by FACS for content of CFSE (Fig. 4C). Cells which have proliferated exhibit less CFSE fluorescence due to intracellular dilution of the stain (26, 36). In accordance with published observations, treatment with CT stimulated depletion of CD8⁺ cells (2, 53), since substantially decreased frequencies of dividing CD8⁺ cells were found in these cultures (~2%). In contrast, dividing CD8⁺ cells were detectable in cultures treated with LT-IIa or LT-IIb at frequencies of ~23% and ~20%, respectively. These frequencies, however, were lower than the frequencies of dividing CD8⁺ cells in untreated control cultures which had been activated with ConA (~45%). LT-IIa(T14I) had little or no effect on ConA-induced CD8⁺ cell proliferation and therefore behaved similarly as in the experiment reported in Fig. 3b (data not shown). Taken together, these results suggest that ConA-mediated activation-proliferation reduced the ability of LT-IIa to stimulate apoptosis of CD8⁺ cells. Decreased susceptibility to LT-IIa-induced depletion, however, was not the result of dilution of the enterotoxin by its binding to GD₁₁₀, since the resistance conferred by activation could be observed at doses of LT-IIa ranging from 1 to 100 μg/ml (data not shown). Since LT-IIa, LT-IIb, and CT differ in their capacity to modulate apoptosis and proliferation of CD8⁺ cells, these results were
consistent with the model that these cellular effects are dependent upon binding of the enterotoxins to different ganglioside receptors.

Activation and proliferation of CD4⁺ cells. To investigate whether the ganglioside binding specificities of LT-IIa, LT-IIb, and CT induce differential modulation of the CD4⁺ T-lymphocyte subset, the effects of these enterotoxins on CD4⁺ T-cell activation and proliferation were examined. Single-cell suspensions from spleens were cultured with ConA for 24 h or 48 h in the presence or absence of LT-IIa, LT-IIb, or CT. The CD4⁺ cells within the splenic cell population were then analyzed by FACS for levels of expression of CD25 and CD69 (Fig. 5A). CD25 and CD69 are cell surface markers which are upregulated on T cells after activation (15). ConA stimulation increased the levels of CD25 and CD69 expressed by CD4⁺ T cells in comparison to expression of those markers in untreated cells. When cells were stimulated with ConA in the presence of CT, however, reduced levels of CD25 and CD69 were observed in comparison to untreated controls. LT-IIa and LT-IIb exhibited little or no effect on the expression of CD25 and CD69 in CT. After 4 days of culture, cells were analyzed by FACS for proliferation by use of CFSE staining (Fig. 5B). CD4⁺ T-cell proliferation was apparent in ConA-stimulated cultures, but not in unstimulated controls (data not shown). Concurrent activation with ConA and CT was associated with a substantially reduced proportion of dividing CD4⁺ T cells in comparison to untreated controls, cultures treated with LT-IIa, or with cultures treated with LT-IIb plus CT. Four division peaks of CD4⁺ cells were evident in each culture treated with any one of the three enterotoxins in the presence of ConA. This pattern suggested that the CD4⁺ cells proceeded through a similar number of divisions regardless of the enterotoxin used for treatment. Interestingly, a minor population of dividing cells (Fig. 5B) comprising about 5% of the entire population was detected in cultures activated with ConA in the presence of CT. Further analysis revealed that these cells expressed the phenotype B220low CD138⁻, which was identical to the phenotype of plasma cells formed following activation of splenic cells with LPS plus CT (see Fig. 6B, below). From this pattern of staining, it was determined that the dividing cells in the CT-plus-ConA-treated cultures were likely to be plasma cells. These results are consistent with a model in which triggering of distinct ganglioside receptors by LT-IIa, LT-IIb, and CT is involved in differential modulation of CD4⁺ T-cell responses and plasma cell induction.

Expression of gangliosides and T-cell cycle. After different numbers of divisions, the surface phenotype of cells is often markedly dissimilar (26, 28). To determine if the enterotoxin-
associated differences in activation, proliferation, and apoptosis induction of T cells were due to modulation of ganglioside receptor expression in correlation with the cell division cycle number. T cells in splenic cell populations were labeled with CFSE, stimulated with ConA for 4 days, and evaluated for expression of specific ganglioside receptors by assessing the patterns of binding to LT-IIa, LT-IIb, and CT. The level of binding of LT-IIb and CT to CD3<sup>H11001</sup>/H11011 T cells did not significantly differ between proliferating and nonproliferating cells (Fig. 5C). LT-IIa binding levels, however, were slightly increased as cells proceeded through various rounds of division. These results indicated that expression of GD1a, GD1b, and GM1 is not substantially affected by cell proliferation. This determination ruled out the possibility that enterotoxin-induced differences in T-cell activation, proliferation, and induction of apoptosis were due to decreased ganglioside expression.

**Proliferation and differentiation of B220<sup>+</sup> cells.** To explore the possibility that B-cell responses were regulated differentially by LT-IIa, LT-IIb, and CT, the effect of these enterotoxins on polyclonal B-cell responses induced by LPS was examined by analyzing B-cell proliferation and differentiation profiles of splenic cell populations (Fig. 6A). While LPS stimulation of splenic cells at day 3 elicited modest proliferation, differentiation of B220<sup>+</sup> cells into plasma cells was not evident. Similar results were obtained when LPS-stimulated cultures were treated with LT-IIa or with LT-IIb, for which only weak proliferation of B220<sup>+</sup> cells was observed, again without induction of differentiation into plasma cells. When cells were activated with LPS in the presence of CT, however, the frequency of dividing B220<sup>+</sup> cells (~56%) was substantially higher than in cultures treated with LT-IIa (~26%), LT-IIb (~16%), or in untreated control cultures (~17%). Additionally, several phenotypic changes that accompany B-cell differentiation into plasma cells (25, 28) (e.g., down-regulation of B220, CD86, and CD40 expression; up-regulation of CD138 and CD80 markers) was observed in a fraction of the cells treated with CT (Fig. 6B), but not in cells treated with either LT-IIa or LT-IIb. Treatment of splenic cells with CT was associated with induction of CD138<sup>H11001</sup>/H11011 B220<sub>low</sub> cells which attained a frequency of ~15% of the total cell population. CD138<sup>H11001</sup>/H11011 B220<sub>low</sub> cells were not detectable in cultures treated with LT-IIa or LT-IIb or in untreated control cultures. Staining for intracellular immunoglobulin confirmed that the cells expressing the CD138<sup>H11001</sup>/H11011 B220<sub>low</sub> phenotype were, indeed, plasma cells (data not shown). Thus, the different capacity of LT-IIa, LT-IIb, and CT to modulate B-cell proliferation and differentiation further supported the hypothesis that the different receptor binding specificities of these toxins may be a strong determinant for their distinct immune regulatory effects on B cells.

**Expression of CD80 (B7-1) and CD86 (B7-2) on B220<sup>+</sup> cells.** The initial step in Ag-specific T-cell activation is modulated by Ag-presenting cells (APC) that, in combination with cell surface costimulatory molecules, adsorb, process, and present Ags on the cell surface in a major histocompatibility complex class II-dependent manner (6). Induction of cellular processes to
increase the efficiency of Ag processing and presentation would be expected to augment Ag-specific immune responses. It was hypothesized, therefore, that the adjuvant properties of the three enterotoxins were mediated by the induction of cellular mechanisms that increased the efficiency of Ag processing and/or presentation. Since the levels of costimulatory molecules on the surfaces of APC influence the induction of the T-cell response and the patterns of cytokines associated with that response (6, 13), the expression levels of costimulatory molecules on B cells were examined after treatment with LT-IIa, LT-IIb, and CT (Fig. 6C). Splenic cell suspensions were stimulated for 24 h with LPS in the presence or absence of each of the three enterotoxins. B220<sup>+</sup> cells within the treated splenic cell population were subsequently analyzed for levels of expression of CD80 and CD86, two costimulatory molecules which are up-regulated after Ag recognition by B220<sup>+</sup> cells (27). As expected, B220<sup>+</sup> cells from LPS-stimulated cultures exhibited enhanced surface expression of CD86 in comparison to expression of CD86 in B220<sup>+</sup> cells from cultures which were unstimulated. Concurrent stimulation with LPS plus CT further enhanced expression of CD86 on B220<sup>+</sup> cells. LT-IIa and LT-IIb, however, exhibited little or no effect on the expression of CD86 by LPS-activated B220<sup>+</sup> cells. While a weak up-regulation of CD80 was observed in B220<sup>+</sup> cells following stimulation with LPS alone, no increase in CD80 expression was observed in cells activated with LPS in the presence of LT-IIa or LT-IIb. CD80 levels were up-regulated by CT, but to a lesser extent compared to the induced levels of CD86.

As each of the enterotoxins had a distinctly different effect
on B-cell modulation, the data were consistent with a model in which the modulatory differences for B cells were correlated with the different ganglioside binding affinities of each of the enterotoxins.

**DISCUSSION**

CT, LT-IIa, and LT-IIb exhibit distinct adjuvant responses and induce discrete Th cytokine production and IgG subclass antibody responses in mice (37, 38). In the present study, it was shown that LT-IIa, LT-IIb, and CT bind differentially to several immune cell types and exert differential effects on (i) apoptosis induction and proliferation of CD8+ T cells; (ii) CD4+ T-cell activation and proliferation; (iii) costimulatory molecule expression by B cells; and (iv) B-cell proliferation and differentiation into plasma cells. From these data, it is clear that LT-IIa, LT-IIb, and CT exhibit different immunomodulatory activities which are correlated at least in part with their respective ganglioside binding specificities.

LT-IIa and CT, but not LT-IIb, induced substantial depletion of resting CD8+ cells. The capacity of LT-IIa and CT to induce death of CD8+ cells may be explained by the enhanced binding of these two enterotoxins to CD8+ lymphocytes: LT-IIa and CT had elevated binding to CD8+ cells compared to binding levels of LT-IIb. In comparison with CD4+ or B220+ cells, CD8+ cells exhibited increased susceptibility to either LT-IIa- or CT-induced depletion. The augmented susceptibility of CD8+ cells to LT-IIa-associated depletion may be related to their potential to bind more than LT-IIa than is bound by CD4+ or B220+ cells. If this were the case, the relative levels of gangliosides expressed by immune cells might determine, in part, their responsiveness to the enterotoxins in regards to immunomodulation. These findings are consistent with published observations which demonstrate increased sensitivity of CD8+ T cells to LT-IB- or CTB-induced cell death. We did not, however, observe any preferential binding of CT to CD8+ cells, as has been previously noticed for binding of the B pentamers of CT (CTB) and the B pentamers of LT-I (LT-IB) to CD8+ cells (2, 49, 53).

The apoptosis-inducing properties of LT-IB had been shown to be critically dependent upon binding of the pentamer to GM1; the mutant LT-IB/G33D, which has no detectable binding affinity for GM1, does not elicit the apoptotic effect (42). Experiments described herein demonstrated that LT-IIa-associated depletion of CD8+ cells is also an apoptotic event. LT-IIa(T34I), which has no detectable in vitro binding affinity for GD1a, GD1b, and GM1, had no capacity to induce apoptosis in CD8+ cells. These data provide direct evidence for the involvement of ganglioside receptor binding in the modulation of lymphocyte survival by LT-IIa and provide the initial data to demonstrate that type II enterotoxins can induce apoptotic activity in lymphocytes.

The differential ability of the three enterotoxins to induce apoptosis and their differing affinities for gangliosides provide a means to determine which of the gangliosides triggered apoptosis induction. For example, in a manner similar to that of CTB and LT-IB, LT-IIa could have induced apoptosis of CD8+ cells via interactions with GM1, one of the three gangliosides to which LT-IIa is known to bind. Blockade of GM1 on the lymphocyte surface with mutant LT-IIa(T14I), which had no ability to induce apoptosis, did not, however, prevent LT-IIa-induced apoptosis of CD8+ cells. This result suggested that gangliosides other than GM1 are likely involved in eliciting the apoptotic effect induced by LT-IIa. Given that LT-IIa has a binding affinity for GD1b which is substantially higher than its affinity for GM1 and that LT-IIb, which binds only to GD1b, did not elicit depletion of CD8+ cells, we posit that binding of LT-IIa to GD1b is the initial trigger for LT-IIa-induced CD8+ apoptosis.

The mechanism by which LT-IIa induces apoptosis in CD8+ cells has not been precisely described on the molecular level. GM1 is known to associate with cholesterol-rich, detergent-insoluble membrane microdomains (lipid rafts) (10, 54), and it is not unreasonable to hypothesize that lipid rafts also contain GM1. Numerous proteins associated with cell signaling are localized within lipid rafts (9, 10). It is conceivable, therefore, that binding of LT-IIa to GD1b within lipid rafts positions the enterotoxin in a manner to interact with signaling molecules that participate in modulation of lymphocyte apoptosis.

Treatment of splenic cell populations or of purified CD8+ cells with LT-IIa or CT was associated with depletion of resting CD8+ cells. While ConA-driven activation and proliferation significantly reduced the susceptibility of these CD8+ cells to LT-IIa-induced depletion, treatment with ConA had no detectable effect on the susceptibility of CD8+ cells to apoptosis induction by CT. These results suggested that activation and proliferation of CD8+ lymphocytes by ConA may alter the sensitivity of those cells for LT-IIa-mediated apoptosis or that activation of B cells or macrophages by ConA plus LT-IIa, but not by ConA plus CT, may provide the costimulatory signals necessary to prevent apoptosis of activated CD8+ cells.

Gamma interferon (IFN-γ) production by CD8+ lymphocytes contributes to the priming of CD4+ T cells for subsequent development into polarized Th1 cells (18, 34). It is possible that induction of apoptosis in CD8+ cells by CT may reduce the levels of IFN-γ and subsequently inhibit the differentiation of CD4+ T cells into Th1 effectors. Conversely, since activated or costimulated CD8+ cells are refractory to LT-IIa-induced apoptosis, production of IFN-γ by these cells may contribute to the priming of CD4+ T cells towards Th1 effectors. With these conjectures in mind, we hypothesize that the differential susceptibility of activated or dividing CD8+ cells to experimental LT-IIa- or CT-induced apoptosis may be one of the major causative events that underlie the distinct patterns of Th responses induced by these two enterotoxins in vivo.

Analysis of enterotoxin-treated splenic cells activated with ConA suggested that CT exhibits distinct suppressive effects on CD4+ T-cell proliferation that were less apparent in cultures treated with either LT-IIa or LT-IIb. As has been demonstrated previously, CT reduced the expression of activation markers on CD4+ T cells (39), but neither LT-IIa nor LT-IIb suppressed CD25 or CD69 expression on these cells. It has been reported that treatment with CT induces apoptosis in ConA-stimulated CD4+ T cells (50) and that expression of surface markers is often down-modulated during apoptosis (32). We did not observe, however, any apoptosis-inducing effect of CT on CD4+ T cells activated with ConA that could explain the observed down-modulation of CD25 and CD69 Ag expression in these cells.

Analysis of enterotoxin-treated splenic cells stimulated with
either ConA or LPS revealed that CT also exerted distinct modulatory effects on B220^+ cells. Our observations also provide evidence for distinct modulatory effects on B220^+/H11001^+ cells treated with either LT-IIa or LT-IIb. Our observations also indicate that CT also exerted distinct modulatory effects on B220^+ cells. Costimulation of B cells via CD86 preferentially stimulates IL-4 production by T cells (13, 27, 31). Thus, the involvement of enhanced frequencies of B cells expressing CD86 on their respective B pentamers differentially induce and regulate cytokine production. This is consistent with the idea that a new enterotoxin of Escherichia coli which activates adenylate cyclase in eukaryotic target cells is not plasmid mediated. Infect. Immun. 52:587–589.


Honda, T., T. Tsuji, Y. Takeda, and T. Miwatani. 1981. Immunological...
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