Induction of Thymus-Derived γδ T Cells by Escherichia coli Enterotoxin B Subunit in Peritoneal Cavities of Mice

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We examined the activation of intraperitoneal T cells in BALB/c mice by the Escherichia coli enterotoxin B subunit, which induced a specific Th2 type of T-cell response to intraperitoneally coadministered bovine immunoglobulin G. The numbers of γδ T cells increased significantly after intraperitoneal administration of the B subunit in a time-dependent manner; these numbers were not affected by the B-subunit G33D mutant, which is defective in GM1 ganglioside-binding ability. Early after administration a small number of γδ T cells produced either interleukin-4 (IL-4) or gamma interferon, while late after administration primarily IL-10-producing γδ T cells were detected. γδ T cells induced by the B subunit did not express a characteristic V gene over the time course of the study. The induction of γδ T cells did not occur in athymic nu/nu mice but could be induced upon transplantation of fetal AKR thymus-like αβ T cells. γδ T cells in athymic nu/nu mice with a fetal thymic graft predominantly expressed the donor Thy-1.1 antigen but not the host Thy-1.2 antigen. The induction of these T cells, however, could not be restored by coadministration of the B subunit with peritoneal cells from normal mice. These results suggest that the B subunit activates intraperitoneal γδ and αβ T cells in a manner dependent upon its ability to bind to GM1 ganglioside. γδ T cells induced by the B subunit are Th2-type cells derived from the thymus. These γδ T cells may be functionally involved in specific Th2 responses to the B subunit, which possibly acts as an adjuvant through the influence of αβ T cells.

In mice, T-cell-receptor (TCR) γδ T cells develop through multiple pathways (23). γδ T cells expressing Vγ5/Vδ1 or Vγ6/Vδ1 first appear in the fetal thymus (7, 11). Vγ8/δ6+ T cells later migrate to the epidermis and exclusively colonize this peripheral site (2, 7). Vγ8/δ6+ T cells also colonize the mucosal epithelia of the tongue, uterus, vagina, and lung (12); but later, they can also distribute to other, peripheral organs (22, 25, 27). The adult thymus can also generate γδ T cells expressing primarily Vγ1 or Vγ4 (11, 24). A third extrathymic developmental pathway for γδ T cells generates γδ T cells preferentially expressing Vγ1, Vγ4, or Vγ7 (16, 24, 35). These pathways create a diverse range of γδ T cells expressing many types of Vγ within the peritoneal cavities of adult mice (20).

Particularly intriguing aspects of γδ T cells are their distinctive localization at sites of infection, in addition to normal epithelial tissues. This association at sites of first contact with infectious agents or their products implicates γδ T cells in frontline defense reactions. The preferential expression of γδ T cells can be due to the selective homing and proliferation of cells controlled by a number of factors unique to γδ T cells and a particular site or inflammatory conditions, such as microbial infection (2).

The contribution of γδ T cells to host defenses against bacteria has been examined by infection of T-cell-depleted mice with intracellular bacteria (4, 17, 28). Mice rendered deficient for γδ T cells by homologous recombination of the TCR-δ chain gene exhibited diminished host defenses against Mycobacterium tuberculosis (17). γδ T cells, however, may have heterogeneous functions during infectious disease. Mice deficient for TCR-δ suffered intestinal damage upon oral infection with Entamoeba vermiciformis, suggesting that intraepithelial γδ T cells function in the resolution of intestinal inflammation (26). Moreover, although protection against extracellular bacteria depends primarily on neutrophils and antibodies (Abs) (32), γδ T cells are also important for host defense against pathogens such as Escherichia coli (20, 21, 30).

Multiple different bacterial antigens, such as mycobacterial purified protein derivative, tetanus toxoid, staphylococcal enterotoxin A, heat shock protein 65, isopentenyl pyrophosphate, and hemolysin from Listeria monocytogenes or E. coli, serve to initiate γδ T-cell-mediated host defenses against bacteria (2). Lipopolysaccharide (LPS), which induces a potent host defense response against E. coli infection through the induction of cytokines such as gamma interferon (IFN-γ) and interleukin-15 (IL-15), also stimulates γδ T cells (20, 21, 30).

Heat-labile enterotoxin (LT) is produced by enterotoxigenic E. coli and causes severe diarrhea in animals and humans in a manner similar to that of cholera toxin (CT), produced by Vibrio cholerae. Both toxins are composed of A and B subunits. The A subunit is toxic, while the B subunit allows the toxin to bind to the GM1 ganglioside. The B subunit exhibits a high level of antigenicity (9); controversy remains, however, as to whether the molecule exhibits an adjuvant action (3, 6, 19, 33) against antigens coadministered nasally, peritoneally, orally, or intramuscularly or whether it has a tolerative effect (1, 29, 34).

In this study, we examined the effect of the B subunit of LT...
(LT-B) on the proliferation of γδ T cells. As the induction of γδ T cells occurs at the site of first contact with infectious agents or their products and the peritoneal cavity of mice is a tissue site for the preferential expression and induction of γδ T cells, we examined whether LT-B might induce γδ T cells by injecting LT-B into the peritoneal cavity. We also evaluated the relationship between γδ T cells activated by LT-B administration and the antigenicity of LT-B and its function as an adjuvant stimulus.

MATERIALS AND METHODS

Mice, plasmonics, and bacteria. All experiments were performed with BALB/c and ICR female mice (Shizuoka Animal Co., Ltd.) aged 4 to 5 months. Age-matched female mice served as controls. The athymic nu/nu mice and fetal AKR mice were purchased from Charles River Co., Ltd.

The Bluescript II SK−/− plasmid carrying the LT-B gene (Toyobo Co., Ltd.) was constructed from strain EWD 299 by PCR, as described previously (14). The G33D B subunit, a mutant form with an aspartate in place of the endogenous glycinine at position 33 from the N terminus, was prepared as described previously (31).

Preparation of normal and mutant LT-B subunits. Recombinant LT-B (rLT-B) was purified by immobilized n-galactose affinity column chromatography in TEAN buffer (50 mM Tris-HCl, 1 mM EDTA, 3 mM NaN3, 0.2 M NaCl [pH 6.8]), as described previously (14). Bacterial culture in CAYE medium (2.0% Casamino Acids, 0.6% yeast extract, 0.25% NaCl, 0.871% K2HPO4, 0.25% glucose, 0.1% trace salt solution [5.0% MgSO4, 0.5% MnCl2, 0.5% FeCl3, 0.0001% H3BO3], isolation of crude cell extracts, and rLT-B purification by successive chromatography steps have been reported previously (14).

The G33D LT-B mutant bound to the GM1 ganglioside receptor at a 10,000-fold lower affinity than normal LT-B, as determined by the GM1 immunosorbent assay. G33D was purified by affinity chromatography with anti-LT-B Abs, as reported previously (31).

Determination of antibody titers in serum. The antibody titers in serum were determined by enzyme-linked immunosorbent assay. Brieﬂy, preimmune and immunized serum were added to the wells. The plates were incubated at 37°C for 60 min. Bound Abs were detected by using酶 alkaline phosphatase-conjugated rabbit anti-mouse IgG, IgG1, IgG2a, IgG2b, and IgG3 Abs at a dilution of 1/1,000 and visualization with 4-nitrophenylphosphate (1 mg/ml). The titer was deﬁned as the absorbance that yielded for each IgG a value of 1.0 above the background value.

Preparation of peritoneal cells. After intraperitoneal (i.p.) injection of rLT-B (20 μg in PBS [pH 7.2]) into mice, we injected 2 ml of RPMI (Gibco BRL, Co., Ltd.) containing 10% fetal calf serum (FCS) and 10 U of heparin per ml into the peritoneal cavity. Peritoneal cells were collected by centrifugation with a swing rotor (Tomy RL-131) at 1,000 rpm for 5 min. After extensive washing in PBS containing 2% FCS, the cells were passed through a nylon filter prior to experimentation.

FCM. The following monoclonal Abs (MAbs) coupled to ﬂuorescein isothiocyanate (FITC), phycoerythrin (PE), or biotin were used for ﬂow cytometry: biotin–anti-CD3ε (clone 145-2C11), FITC–anti-mouse αβ TCR (clone H57-597), FITC–anti-mouse γδ TCR (clone GL3), PE–anti-IL-4 (clone 11B11), PE–anti-mouse IFN-γ (clone XMG1.2), PE–anti-mouse IL-10 (clone JES5-2A5), PE–anti-mouse Thy-1.1 (clone H1S1), and PE–anti-mouse Thy-1.2 (clone 53-2.1). Anti-Thy-1.1 and anti-Thy-1.2 clones were purchased from Bioscience Co., Ltd. Additional MAbs were purchased from Pharmingen Co., Ltd. Species and isotype-matched IgGs coupled to FITC, PE, or biotin (Bioscience or Pharmingen Co., Ltd.) were used as staining controls.

To stain the surface antigens on peritoneal cells, 107 cells were incubated with 5% skim milk at 4°C for 30 min. After washing in PBS containing 2% FCS, the samples were stained with a mixture of the designated anti-CD IgG MAb at 4°C for 30 min, as described previously (13). For intracellular cytotoxic staining, we added 2 ml of ﬂuorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson) and incubated for 10 min at room temperature. Following a wash in PBS containing 2% FCS, the samples were stained with the designated MAb at 4°C for 30 min. Flow cytometric analysis was performed with a FACSscan instrument (FACSCaliber; Becton Dickinson); the data were analyzed with Lyss II software.

Analysis of V-gene segment use by reverse transcription-PCR (RT-PCR).

FIG. 1. Serum IgG subclass response to LT-B and BGG in ICR mice immunized once i.p. with LT-B and BGG, LT-B (10 μg) and BGG (100 μg) were coadministered via the i.p. route to six ICR female mice. The mice were bled 4 weeks after the vaccination. The anti-LT-B and BGG titers were defined with an LT-B- or BGG-coated enzyme-linked immunosorbent assay plate. Values represent mean ± standard deviations. Asterisks indicate that the values are significantly greater (P < 0.05) for the LT-B- and BGG-treated groups than for the control group or the group treated with BGG alone. The data shown are representative of two independent analyses with six mice, with similar results obtained in each analysis.

Total RNA was extracted from mouse peritoneal cells by the acid-guanidinium-phenol-chloroform method. DNA synthesis was performed by PCR. RNA was primed by using a cDNA cycle kit (Invitrogen Corp., San Diego, Calif.) with 10 μg of total RNA in the presence of either 20 pmol of the γ-chain C-region (cy) primer (5′-CCT ATG GAG GAT TTG TTG TAT CAG C-3′) or 6.7 pmol of the δ-chain J-region (3β) primer (5′-TTC GGT TCT CCA CAG CAG TCT GG-3′) in 21-μl reaction mixtures. PCR was performed with a PCR thermal cycler (Takara Corp., Tokyo, Japan), with PCR cycles composed of 1 min at 94°C, 1 min at 54°C, and 30 s at 72°C. Prior to the first cycle, the samples were incubated for 7 min at 94°C. After 35 cycles, extension was performed for an additional 4 min at 72°C. The 5′-V-gene-specific primers have been described previously (30) and are as follows: Vγ1/2, 5′-ACA CAG CTA ACC ATT GGT AC-3′; Vγ2, 5′-CGG CAA
The absolute numbers of peritoneal cells and γδ and αβ T cells after i.p. injection of LT-B or G33D into six BALB/c mice. Peritoneal cells obtained from mice for each set of experimental conditions were pooled, stained with the designated MAb, and then analyzed by flow cytometry. The means and standard errors for six mice are shown. Asterisks indicate the significance of differences in the values for each condition for the mice at day 0 (P < 0.05) (*), and mice injected with G33D (P < 0.05) (**).
G33D increased the numbers of peritoneal cells and both types of T cells, there was no difference in the levels of induction of these cells by 20 and 40 μg. As LT-B appears to induce the influx of peritoneal γδ T cells, we characterized the γδ T cells recruited by LT-B.

Dose-response of absolute numbers of peritoneal γδ T cells after i.p. injection of each dose of LT-B. We examined the responses of γδ T cells to increasing doses of LT-B (Fig. 4). Following injection, the absolute numbers of γδ T cells increased proportionately in a dose-dependent manner. Treatment of LT-B-treated samples with 10 μg of polymyxin B per ml, a quantity capable of neutralizing more than 10 μg of LPS per ml, did not affect the induction of γδ T cells. Treatment of LT-B at 100°C for 10 min reversed the induction of γδ T cells in the peritoneal cavity, suggesting that the LT-B itself and not the contaminating LPS induces the recruitment of peritoneal γδ T cells.

Cytokine production by γδ T and αβ T cells in the peritoneal cavity induced by LT-B. To elucidate the function of γδ T cells induced by LT-B, we examined the production of cytokines, such as IL-4, IL-10, and IFN-γ, by γδ T cells. Following intracellular cytokine staining, a small number of γδ T cells stained with anti-IL-4 and anti-IFN-γ Abs at 3 days. At 5 and 7 days, however, T cells stained with anti-mouse IL-4 and IL-10 Abs, respectively (Fig. 5), demonstrating a change in the quality of the T-cell response.

Moreover, we also examined the pattern of cytokine production by αβ T cells induced by LT-B (Fig. 6). αβ T cells produced cytokines identical to those synthesized by γδ T cells.
Vγ2- and Vδ2-gene expression by γδ T cells in the peritoneal cavities of mice injected i.p. with LT-B. To examine V-gene expression by γδ T cells in the peritoneal cavity following LT-B injection, we extracted total RNA from peritoneal cells. V-gene expression was analyzed by RT-PCR. γδ T cells from the peritoneal cavities of mice injected with PBS exhibited bands corresponding to Vγ1/2, Vγ2, Vγ4, and Vγ6. γδ T cells recruited following LT-B administration demonstrated the same bands at 1, 3, 5, and 7 days after LT-B injection. DNA sequencing of each band confirmed that these sequences corresponded to portions of the Vγ1/2, Vγ2, Vγ4, and Vγ6 genes, as described previously (8).

Bands related to V61, V64, V65, V66, and V67 were detected from RNA prepared from peritoneal cells from normal mice. At 1 day after LT-B administration, however, the amount of a band representing Vγ6 (Vγ6/δ1) could be seen to be relatively increased. However, there was not a characteristic usage of the Vδ gene over the time course of the study. The small number of peritoneal γδ T cells recruited by G33D administration showed that Vγ and Vδ usage was identical to that seen in normal mice.

Changes in numbers of peritoneal γδ T cells following LT-B stimulation in vitro. To elucidate whether LT-B acts directly on the proliferation of peritoneal γδ T cells or indirectly through the stimulation of other peritoneal cells, we examined the changes in the numbers of γδ T cells in vitro following stimulation with LT-B. LT-B did not affect the numbers of γδ T cells over a 7-day period of stimulation.

Lack of induction of γδ and αβ T cells by injection of LT-B into athymic nu/nu mice and restoration of induction by AKR fetal thymus graft. γδ T cells develop through multiple pathways, and these pathways are both dependent and independent of thymic selection. We therefore determined the effect of the thymus on the induction of γδ and αβ T cells following i.p. injection of LT-B into athymic nu/nu mice. LT-B was unable to induce the accumulation of either type of T cell in the peritoneal cavities of athymic nu/nu mice. Upon transplantation of fetal ARK thymus tissue into BALB/c nu/nu mice, however, the induction of γδ and αβ T cells following LT-B stimulation could be partially restored in the mice. The γδ-type T cells used Vγ2 and Vδ1, identical to those seen in the normal mice.

Moreover, both T-cell subsets induced by LT-B expressed the Thy-1.1 antigen derived from the donor AKR mice but did not express Thy-1.2, which is expressed by the recipient BALB/c mice (Fig. 7).

No induction of γδ or αβ T cells in athymic nu/nu mice by coinjection of LT-B and peritoneal cells. We examined the induction of γδ T cells from extrathymic tissues by LT-B by coinjection of normal peritoneal cells. When normal peritoneal cells prepared from BALB/c mice (Fig. 8a) or AKR mice (Fig. 8b) were coinjected i.p. into BALB/c nu/nu mice, γδ T cells were not induced by LT-B in either mouse strain. Upon i.p. injection of peritoneal cells from AKR mice treated with LT-B into BALB/c nu/nu mice, peritoneal αβ and γδ T cells expressed the Thy-1.1 antigen derived from the donor AKR mice (Fig. 8c).

**DISCUSSION**

γδ T cells participate in early responses to bacterial infection and play a protective role against pathogens such as *L. monocytogenes*, *Salmonella*, *Mycobacterium*, *E. coli*, and *Toxoplasma gondii* (2, 4, 17, 20, 21, 28, 30). In such intracellular or extracellular bacterial infections, several antigens, including bacterial components and toxins, activate γδ T cells (2). LT and CT,
produced by enterotoxigenic *E. coli* and *V. cholerae*, respectively, induce diarrhea, but they also serve as strong adjuvants that stimulate immune responses to other antigens influencing the αβ T- and B-cell repertoire (3, 19, 33). The B subunits of these toxins alone are strongly antigenic and appear to have some adjuvant action, but this effect is highly reduced from that seen for the intact holotoxin. Kim et al. (15) reported that holo-LT selectively inhibits the extrathymic T-cell development of intestinal epithelial lymphocytes, including αβ and γδ T cells. As yet, however, no reports have demonstrated that CT, LT, or their corresponding B subunits can recruit and activate peritoneal γδ T cells. In this study, therefore, we examined the effect of LT-B on peritoneal γδ T cells.

Upon i.p. injection of LT-B into mice, both the percentages and the absolute numbers of γδ and αβ T cells increased in a time-dependent manner (Fig. 4). These increases were dependent on LT-B binding to GM1 ganglioside (Fig. 3). Our analysis of γδ T cells revealed that this population expressed the same Vγ and Vδ genes as peritoneal γδ T cells without LT-B injection. Coadministration of *E. coli* (MV1184 strain) and LT-B, prepared for these studies, to mice did not affect the *E. coli* infection in the peritoneal cavity or the spleen (data not shown). Thus, the γδ T cells induced by LT-B are heterogeneous and do not appear to be a primary defense that mini-

**FIG. 7.** Detection of Thy-1.1 and Thy-1.2 antigens on γδ and αβ T cells induced by rLT-B in *nu/nu* mice grafted with fetal AKR thymus tissue. Three days after three *nu/nu* mice grafted with fetal thymus tissue were injected i.p. with PBS (closed areas) or rLT-B (20 μg; open areas); and peritoneal cells were isolated, stained with anti-Thy-1.1 or anti-Thy-1.2 Abs, and then analyzed by flow cytometry. We analyzed the positive cells for cells double positive for CD3γδ and γδ or αβ. Representative data from two independent analyses are shown, with similar results obtained in each analysis.

**FIG. 8.** Lack of induction of γδ or αβ T cells by rLT-B in athymic *nu/nu* mice by i.p. injection of peritoneal cells from normal mice. Normal peritoneal cells were obtained from either three BALB/c mice (a) or three AKR mice (b and c). Normal peritoneal cells were coadministered with PBS (left) or rLT-B (right) (20 μg) to three BALB/c *nu/nu* mice. Three days after injection, pooled cells were stained with labeled anti-CD3, anti-TCR-γδ, or anti-TCR-αβ (a and b) and anti-Thy-1.1 or Thy-1.2 (c) MAbs and analyzed by flow cytometry. The results are representative of those from two independent experiments, with similar results obtained in each experiment.
izes E. coli infection, although LT-B is an agent that induces γδ T cells in the frontline immune response.

Functionally, LT-B-induced γδ T cells produced cytokines of the Th2 type, such as IL-4 and IL-10, like αβ T cells do (Fig. 5 and 6). IL-4 is a key cytokine that shifts the Th1-Th2 balance toward Th2-type cells. IL-10 inhibits the production of IL-12 and IFN-γ, key cytokines that shift the Th1-Th2 type balance to a Th1-like response, and promotes the activation of Th2-type cells (10, 18). As γδ T cells have also been divided into at least two subsets (Th1 and Th2) (5), LT-B shifts the Th1-Th2 balance of γδ T cells to the Th2-type response (Fig. 5). Moreover, significant IgG1 and IgG2b Ab responses but not IgG2a Ab responses to LT-B and BGG were observed by i.p. injection of LT-B and BGG (Fig. 1). Thus, the Th2 types of γδ and αβ T cells induced by LT-B may work together, promote the humoral immune response to LT-B itself, and allow LT-B to exhibit its adjuvant action to induce a specific Th2 type of response to BGG.

Next, we examined whether γδ T cells induced by LT-B might originally be thymic or extrathymic. We first examined the stimulatory effect of LT-B on peritoneal cells in vitro. γδ T cells did not proliferate upon in vitro stimulation of peritoneal cells by LT-B, suggesting that LT-B induces the influx of γδ T cells into the peritoneal cavity. γδ T cells could not be induced by LT-B administration in athymic nu/nu mice; induction was restored upon grafting of a normal fetal thymus. Thus, the induction of γδ T cells by LT-B is dependent on the thymus. Moreover, γδ T cells in athymic nu/nu BALB/c mice with a fetal thymic graft expressed the Thy-1.1 antigen derived from the donor thymic graft, not the Thy-1.2 antigen expressed by the host BALB/c mice (Fig. 7). These data suggest that γδ T cells migrate from the thymus and home into the peritoneal cavity following LT-B stimulation but cannot be derived from extrathymic tissues.

In conclusion, LT-B induces the accumulation of peritoneal γδ T cells in a manner dependent on its binding to the GM-ganglioside receptor. LT-B-induced γδ T cells derived from the thymus produce Th2-type cytokines. These cells may be functionally involved in the promotion of specific humoral immune responses to LT-B and the adjuvant action that promotes the response of specific Th2-type T cells to coadministered antigens through their various interactions with αβ T cells.

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REFERENCES

1. Bergerot, L., C. Ploix, J. Petersen, Y. Moulin, C. Rask, N. Fabien, M. Lindblad, A. Mayer, C. Czereksny, J. Holmgren, and C. Thivolet. 1997. A choleratoilet-insulin conjugate as an oral vaccine against spontaneous autoimmune diabetes. Proc. Natl. Acad. Sci. USA 94:4610–4614.
2. Born, W., C. Cady, J. Jones-Carson, A. Mukasa, M. Lahn, and R. O'Brien. 1995. Immunoregulatory functions of gamma delta T cells. Adv. Immunol. 57:77–144.
3. Elson, C. O., and W. Ealding. 1984. Cholera toxin feeding did not induce oral tolerance in mice and abrogated oral tolerance to an unrelated protein antigen. J. Immunol. 133:578–583.
4. Emoto, M., H. Danbara, and Y. Yoshikai. 1992. Induction of gamma/delta T cells in murine salmonellosis by an avirulent but not by a virulent strain of Salmonella choleraesuis. J. Exp. Med. 176:363–372.
5. Ferrick, D. A., M. D. Schrenzel, T. Mulvania, R. Hsieh, W. G. Ferlin, and H. Lepper. 1995. Differential production of interferon-gamma and interleukin-4 in response to Th1- and Th2-stimulating pathogens by gamma delta T cells in vivo. Nature 373:255–257.
6. Haag, L., W. E. Verweij, C. H. Ladel, C. Ploix, R. Brands, G. J. van Scharrenburg, A. M. Palache, E. Agerstibbe, and J. Wilschut. 2001. Nasal or intranasal immunization of mice with influenza subunit antigen and the B subunit of Escherichia coli heat-labile toxin induces IgA- or IgG-mediated protective systemic immunity. Vaccine 19:2898–2907.
7. Havran, W. L., and J. P. Allison. 1988. Developmentally ordered appearance of tymphocytes expressing different T-cell antigen receptors. Nature 335:443–445.
8. Hayday, A. C. 2000. γδ T cells: a right time and a right place for a conserved third way of protection. Annu. Rev. Immunol. 18:975–1026.
9. Holmgren, J. 1981. Actions of cholera toxin and the prevention and treatment of cholera. Nature 292:413–417.
10. Hsieh, B., M. D. Schrenzel, T. Mulvania, H. D. Lepper, L. DiMoffet-Landon, and D. A. Ferrick. 1996. In vivo cytokine production in murine listeriosis. Evidence for immunoregulation by gamma delta T cells. J. Immunol. 156:232–237.
11. Ito, K., M. Bononnette, Y. Takagaki, N. Nakashishi, O. Kanagawa, E. G. Krocko, and S. Tonegawa. 1989. Different gamma delta T-cell receptors are expressed on tymphocytes at different stages of development. Proc. Natl. Acad. Sci. USA 86:6331–6335.
12. Itohara, S., A. G. Farr, J. L. Lafaille, M. Bononnette, Y. Takagaki, W. Haas, and S. Tonegawa. 1992. Development of a gamma delta thymocyte subset with homogeneous T-cell receptors to mucosal epidermal. Nature 354:754–757.
13. Kawamoto, Y., K. Sasaki, Y. Kato, K. Kojima, T. Tsuji, and A. Miyama. 1996. Rapid killing of murine lymph node T blasts by intestinal intraepithelial lymphocytes in vivo. J. Immunol. 156:653–658.
14. Kawai, H., M. Kato, S. Imamura, T. Tsuji, and A. Miyama. 1996. The amino acids of Escherichia coli enterotoxin B subunit involved in binding to Bio-Gel A-5m or to the glycopeptidase from mouse intestinal epithelial cells. Can. J. Microbiol. 42:983–988.
15. Kim, J. K., I. Takahashi, Y. Kai, and H. Kiyono. 2001. Influence of enterotoxin on mucosal intranet: selective inhibition of extrathymic T cell development in intestinal intraepithelial lymphocytes by oral exposure to heat-labile toxin. Eur. J. Immunol. 31:2960–2965.
16. Kyes, S., E. Carew, S. R. Carding, C. A. Janeway, Jr., and A. Hayday. 1989. Diversity in T-cell receptor gamma gene usage in intestinal epithelium. Proc. Natl. Acad. Sci. USA 86:5527–5531.
17. Kiel, T., Y. Lebbe, C. H. Blum, A. Dreher, K. Reifenberg, and S. H. Kaufmann. 1995. Protective role of gamma/delta T cells and alpha/beta T cells in tuberculosis. Eur. J. Immunol. 25:2877–2881.
18. Klineg, M., A. Richter, and A. Radbruch. 2002. Cytokine memory of T helper lymphocytes. Adv. Immunol. 80:115–181.
19. Lycke, N., T. Tsuji, and J. Holmgren. 1992. The adjuvant effect of Vibrio cholerae and Escherichia coli heat-labile enterotoxins is linked to their ADP-ribosyltransferase activity. Eur. J. Immunol. 22:2277–2281.
20. Matsuomaki, G., R. Takada, and K. Nomoto. 1999. Escherichia coli infection induces only fetal thymus-derived gamma delta T cells at the infected site. Eur. J. Immunol. 29:3877–3886.
21. Morin, J., Y. Tsuchida, Y. Kato, and S. Koyama. 1981. Actions of cholera toxin and the prevention and treatment of cholera. Nature 292:413–417.
30. Takano, M., H. Nishimura, Y. Kimura, Y. Mokuno, J. Washizu, S. Itohara, Y. Nimura, and Y. Yoshikai. 1998. Protective roles of γδ T cells and interleukin-15 in *Escherichia coli* infection in mice. Infect. Immun. 66:3270–3278.

31. Tsuji, T., T. Honda, T. Miwatani, S. Wakabayashi, and H. Matsubara. 1985. Analysis of receptor-binding site in *Escherichia coli* enterotoxin. J. Biol. Chem. 260:8552–8558.

32. Tsuru, S., K. Nomoto, M. Mitsuyama, Y. Zinnaka, and K. Takeya. 1981. Importance of polymorphonuclear leucocytes in protection of mice against *Escherichia coli*. J. Gen. Microbiol. 122:335–338.

33. Williams, N. A., T. R. Hirst, and T. O. Nashar. 1999. Immune modulation by the cholera-like enterotoxins: from adjuvant to therapeutic. Immunol. Today 20:95–101.

34. Williams, N. A., L. M. Stasiuk, T. O. Nashar, C. M. Richards, A. K. Lang, M. J. Day, and T. R. Hirst. 1997. Prevention of autoimmune disease due to lymphocyte modulation by the B-subunit of *Escherichia coli* heat-labile enterotoxin. Proc. Natl. Acad. Sci. USA 94:5290–5295.

35. Yoshikai, Y., M. D. Reis, and T. W. Mak. 1986. Athymic mice express a high level of functional gamma-chain but greatly reduced levels of alpha- and beta-chain T-cell receptor messages. Nature 324:482–485.