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MUCOSAL T CELLS INDUCE SYSTEMIC ANERGY FOR ORAL TOLERANCE

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Heat-labile enterotoxin (LT) from enterotoxigenic *Escherichia coli* is known to possess strong immunoregulatory potential in terms of inhibition of the induction of oral tolerance and adjuvanticity in oral immunization. We found that oral administration of an immunogenic peptide of LT [LT-B(26-45); spanning the residues 26-45 of LT-B] induced systemic unresponsiveness in BALB/c mice resulting in diminished serum IgG responses. It was also shown that the spleen (SP) $CD4^+$ T cells of tolerized mice failed to proliferate, whereas the Peyer's patches (PP) $CD4^+$ T cells responded to the peptide. RT-PCR revealed that the SP $CD4^+$ T cells did not generate IL-2 mRNA, while the PP $CD4^+$ T cells expressed significant levels of IFN-γ, IL-2, IL-4, and TGF-β mRNA. Adoptive transfer of LT-B-specific intraepithelial lymphocytes to the tolerant mice abrogated the tolerance. In the reversed mice, LT-B(26-45)-stimulated SP $CD4^+$ T cells expressed significant levels of IFN-γ, IL-2, IL-4, and IL-6 mRNA. These results indicate that PP $CD4^+$ T cells induce oral tolerance due to systemic T cell anergy.

Heat-labile enterotoxin (LT) produced by *Escherichia coli* strains has been shown to exhibit a potent immunogenicity in mice for the induction of antigen-specific secretory IgA (S-IgA) and serum antibody responses (1). Further, LT is a strong adjuvant that enhances both serum and mucosal antibody responses to unrelated antigens when given by the oral route (1, 2). We have examined molecular mechanisms of immunogenicity and adjuvanticity of LT, revealing that the systemic and mucosal antibody responses induced by LT to itself or to the unrelated antigens are regulated by $CD4^+$ T cells of both T helper cell type 1 (Th1)- and Th2-types (3-5). Epitope mapping of the LT-B molecule revealed that an immunodominant T and B cell epitope spanned residues 36 to 45 of the LT-B molecule (6).

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Further, oral immunization with a 20-mer peptide spanning residues 26-45 induced significant S-IgA and serum IgG responses to the LT-B. Based on these findings, we suggest that the 20-mer peptide may be a good oral vaccine candidate for infection to enterotoxigenic *Escherichia coli* (4).

Presenting antigens by the oral route is a biologically relevant method of inducing antigen-specific peripheral tolerance. Previous studies have shown that one of the mechanisms of the oral tolerance following oral administration of a protein antigen is due to generation of regulatory T cells in the gut-associated lymphoid tissue (GALT) that mediate systemic unresponsiveness (7). Miller et al. (8) reported that $CD8^+$ T cells secreting transforming growth factor-β (TGF-β) were involved in myelin basic protein (MBP)-induced oral tolerance, while other investigators (9) isolated $CD4^+$ T cell clones from mesenteric lymph nodes of SJL mice orally tolerized with MBP. These $CD4^+$ T cell clones were characterized as T helper cell type 2 (Th2) clones, which produced TGF-β together with various levels of interleukin-4 (IL-4) and IL-10. It was also demonstrated that these $CD4^+$ Th2 cells suppressed experimental autoimmune encephalomyelitis (EAE) induced with MBP, suggesting that mucosally derived $CD4^+$ T cells producing mainly TGF-β may have an important function in maintaining oral tolerance.

We report here the generation of oral tolerance by the immunogenic 20-mer peptide of LT-B and mechanisms by which the oral tolerance is induced and/or abrogated.

MATERIALS AND METHODS

Mice: Female BALB/c mice, 6 weeks of age, purchased from Charles River Japan (Atsugi City, Japan) were used.

Induction and abrogation of oral tolerance: Oral tolerance was induced by a multiple low-dose feeding regimen. LT-B(26-45) (Ser-Tyr-Thr-Glu-Ser-Met-Ala-Gly-Lys-Arg-Glu-Met-Val-Ile-Ile-Thr-Ile-Lys-Ser-Gly) was synthesized by the solid-phase procedure (10). The peptide included residues 26 to 45, based on the amino acid sequence (6) of the LT-B molecule. Each mouse was fed 50 μg of LT-B(26-45) dissolved in 0.25 ml of phosphate-buffered saline (PBS) by gastric intubation every seven days for a total of four times. Seven days after the last feeding, mice received an intravenous injection of 50 μg of the peptide in 0.25 ml of PBS. On days 14, 21, 28, and 42 after the first oral feeding, fecal extracts and serum samples were obtained from individual mice, monitored for anti-LT-B(26-45) antibodies by enzyme-linked immunosorbent assay (ELISA). As a control, BALB/c mice were immunized intravenously with 50 μg of LT-B(26-45) in 0.25 ml PBS. One week after the systemic immunization, serum samples were collected and used for monitoring LT-B(26-45)-specific antibody titers.

For abrogation of oral tolerance (11), intraepithelial lymphocytes (IEL) were isolated from BALB/c mice orally immunized with 10^{10} cells of an attenuated *Salmonella typhimurium* strain, EL23 (12), carrying the gene for the LT-B. Four days after the immunization, IEL were isolated as described previously (11). IEL were also isolated from mice immunized orally with 10^{10} cells of *Salmonella typhimurium* SL1438, a recipient for a plasmid coding for LT-B (12). These IEL (1×10^6 cells) were then adoptively transferred intraperitoneally to the mice that had been orally tolerized to the peptide. The tolerized mice then received a subcutaneous injection of 50 μg of the peptide in 0.25 ml of PBS emulsified in an equal volume of complete Freund adjuvant (Difco, Detroit, MI), and serum antibody titers of these mice were determined one week after.

ELISA: Antibody titers in serum and fecal extracts were determined by ELISA. ELISA plates (Nunc) were coated with 100 μl of 50 μg/ml of synthetic peptide [LT-B(26-45)] in PBS. Serial two-fold dilutions of murine sera and fecal extracts were added (100 μl/well). After 2 h incubation at 37°C, unbound antibodies were removed, and alkaline phosphatase-

conjugated goat anti-mouse μ, γ, γ1, γ2α, γ2β, γ3, α heavy chain (Southern Biotechnology Associates, Birmingham, AL) was added. The plates were incubated at 37°C for 2 h, and developed with *p*-nitrophenyl phosphate (1 mg/ml: Wako, Osaka, Japan) in 10 mM diethanolamine buffer (pH 9.6). End point titers were expressed as the reciprocal of the maximum dilution which gave an optical density at 405 nm (OD_{405}) of 0.1.

***In vitro* stimulation of LT-B(26-45)-specific T cells:** Groups of 5 BALB/c mice were orally tolerized by four times consecutive feeding with LT-B(26-45). One week after the last feeding, SP and PP were aseptically removed. Single cell suspensions of splenocytes were prepared by a mechanical disruption, while cell suspensions of PP were obtained by an enzymatic dissociation method as described previously (13). The cell suspensions were washed extensively and resuspended in RPMI 1640 (Sigma) supplemented with sodium bicarbonate, HEPES (15 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamycin (100 μg/ml). The $CD3^+$ T cells were enriched by negative panning on Petri plates coated with goat anti-mouse IgG $F(ab')_2$ (20 μg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA). $CD4^+$ T cells were further purified by positive selection with MACS magnetic beads (Miltenyi Biotec, Sunnyvale, CA). The mAb specific for CD4 (clone GK1.5, American Type Culture Collection, Rockville, MD) was purified from ascites through a protein G column (Pharmacia) and added to the cell suspended at a ratio of 0.5 μg per 10^6 cells. The separated $CD4^+$ T cell population was more than 99% $CD4^+$ $CD8^-$ as determined by direct flow cytometry. The $CD4^+$ T cells (1×10^5/well) were cultured in flat-bottom 96-well microculture plates (Corning) in the presence of mitomycin C-treated splenic feeder cells (1×10^4 cells/well) and 10 μg/well of LT-B(26-45) for 3 days. Analysis of peptide-specific $CD4^+$ T cell proliferation responses was assessed by addition of 0.5 μCi of [^{3}H]thymidine (ICN radiochemicals, Irvine, CA) 6 h prior to cell harvest. Proliferation was determined by liquid scintillation counting. Control experiments were done using $CD4^+$ T cells and feeder cells without peptides. Results are expressed as stimulation index (cpm [^{3}H]thymidine uptake with stimulant / cpm [^{3}H]thymidine uptake in medium only).

Cytokine-specific PCR analysis: Reverse transcription (RT)-PCR analysis was used to detect antigen-specific cytokine mRNA expression by using RT-PCR Amplimer Sets (CLONETECH, Palo Alto, CA). PP were carefully removed from orally tolerant BALB/c mice, while SP were removed both from orally tolerant and tolerance-abrogated mice. $CD4^+$ T cells were isolated from the PP and SP as described above. The $CD4^+$ T cells were cultured with splenic feeder cells and/or the peptide for 3 days. To isolate RNA from the peptide-stimulated or non-stimulated $CD4^+$ T cells, TRIzol reagent (GIBCO BRL , Gaithersburg, MD) was used. RT was carried out using oligo (dT)16 primer, and the specific cDNA fragment was amplified by using cytokine-specific primers and *Taq* DNA polymerase (Roche Molecular Systems, Branchburg, NJ). PCR products were separated by electrophoresis in 2% agarose gels.

RESULTS

Induction of systemic unresponsiveness to LT-B(26-45). Mice immunized orally with LT-B(26-45) two times at a 7 day interval developed clear LT-B(26-45)-specific serum IgG responses, which were comparable to those in mice immunized once intravenously with the peptide (Fig. 1). However, when the mice fed the peptide four times at weekly intervals, the serum IgG responses were markedly suppressed (Fig. 1). On the other hand, mice fed the peptide twice developed enhanced fecal IgA responses specific for the peptide, and high levels of specific fecal IgA responses still remained even after the four consecutive feedings (results not shown). These results indicate that low-dose, multiple feedings of the peptide [LT-B(26-45)] in mice induced systemic unresponsiveness to the relevant antigen, whereas enhanced mucosal antibody response continued to be essentially unaltered.

LT-B(26-45)-specific T helper cell responses in tolerized mice. PP $CD4^+$ T cells from orally tolerant mice significantly proliferated upon stimulation of the peptide,

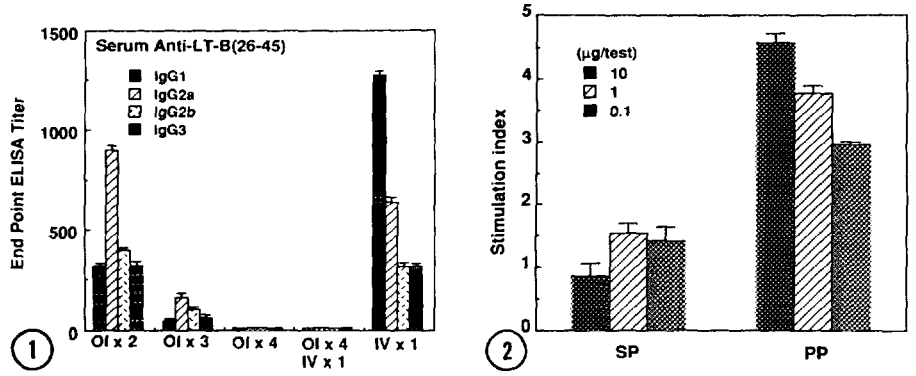

Fig. 1. Induction of systemic tolerance in BALB/c mice by oral immunization with LT-B(26-45). Seven days after the final immunization (OI, oral immunization; IV, intravenous immunization), serum samples were obtained from individual mice and monitored for anti-LT-B(26-45) antibodies by ELISA. Data are expressed as means ± standard deviations of the reciprocal of the maximum dilution which gave an OD_{405} of 0.1.

Fig. 2. T cell responses to LT-B(26-45) in BALB/c mice that had been orally tolerized to the peptide. The stimulation index was calculated as follows: cpm in the presence of the peptide / cpm in the absence of the peptide (2840 ± 265 for SP and 12530 ± 1120 for PP). Data are expressed as means ± standard deviations for triplicate cultures.

while SP $CD4^+$ T cells from the tolerant mice failed to respond to the peptide (Fig. 2). We next examined the cytokine gene expression profile in LT-B(26-45)-stimulated PP $CD4^+$ T cells from the mice orally tolerized with the peptide. When PP $CD4^+$ T cells from the tolerant mice were cultured *in vitro* with the relevant antigen, these cells synthesized significant levels of interferon-γ (IFN-γ), IL-2, and TGF-β mRNA, and a lower level of IL-4 mRNA (Fig. 3). The cytokine mRNA expression was dependent on specific

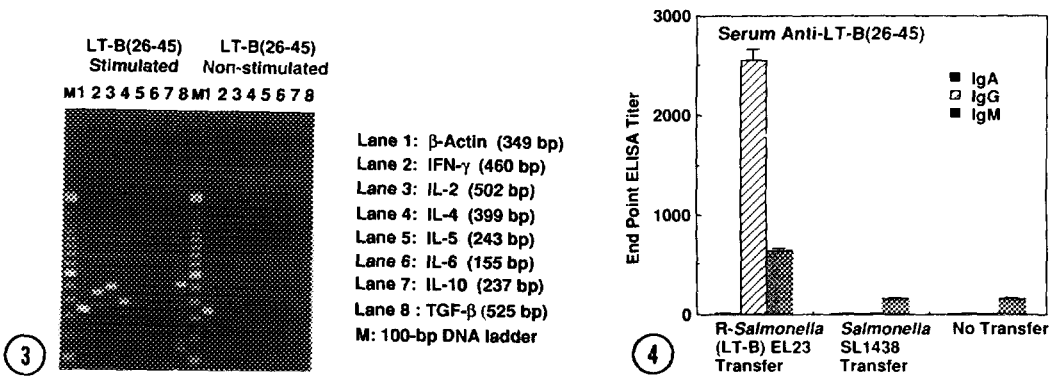

Fig. 3. PCR analysis of cytokine-specific mRNA in LT-B(26-45)-stimulated $CD4^+$ T cells from PP of mice that had been orally tolerized to the peptide. Seven days after the intravenous immunization, $CD4^+$ T cells were isolated and stimulated *in vitro* with the peptide for 3 days. The RNA was extracted from the cells, reverse-transcribed, and amplified by cytokines-specific 5' and 3' primers by using PCR.

Fig. 4. LT-B-specific IEL abrogate systemic unresponsiveness to LT-B(26-45). IEL were adoptively transferred to orally tolerant mice intraperitoneally, and then immunized with the peptide subcutaneously. One week later, LT-B(26-45)-specific antibodies were monitored by ELISA.

stimulation *in vitro* with the peptide. Thus, in addition to the production of mRNA of T helper cell type 1 cytokines, oral tolerance induced by the LT-B(26-45) preferentially involved the generation of cells that produced TGF-β mRNA.

Abrogation of oral tolerance by adoptive transfer of IEL. Since our previous studies revealed that a peptide mimicking the residues 36-45 of the LT-B molecule recognized antibodies elaborated against recombinant *Salmonella*-LT (3, 4), we isolated IEL from mice immunized orally with the recombinant *Salmonella*-LT-B. When LT-B-specific IEL were adoptively transferred to mice that had been orally tolerized by the relevant peptide, conversion of oral tolerance to IgG and IgM responses was seen (Fig. 4). On the other hand, non-antigen-specific IEL from mice orally immunized with *Salmonella typhimurium* SL1438 did not abrogate oral tolerance.

Cytokine gene expression profile of SP CD4+ T cells. SP CD4+ T cells from orally tolerant mice and cultured *in vitro* with LT-B(26-45) failed to produce IL-2, IL-4, and IL-5 mRNA (Fig. 5). However, SP CD4+ T cells from the oral tolerance-abrogated mice expressed IL-2 and IL-4 mRNA.

DISCUSSION

A unique feature of the mucosal immune system is the maintenance of appropriate IgA responses to orally encountered antigens at mucosal surfaces in the presence of systemic unresponsiveness to these antigens, commonly termed oral tolerance. We demonstrated here that mucosally derived CD4+ T cells producing IFN-γ, IL-2, IL-4, and TGF-β can induce systemic unresponsiveness to the orally administered segment peptide

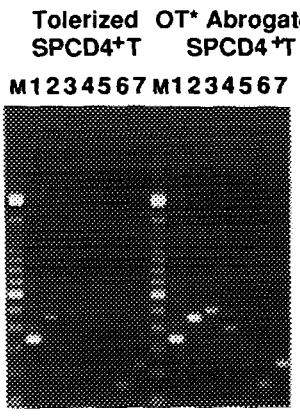

Fig. 5. PCR analysis of cytokine-specific mRNA in LT-B(26-45)-stimulated SP CD4+ T cells both from orally tolerant and tolerance-abrogated mice.

of LT. This cytokine profile is clearly distinct from the classical Th cell clones which include Th1-type (IFN-γ and IL-2-producing) and Th2-type (IL-4, IL-5, IL-6, and IL-10 producing) cells (14). We also analyzed the characteristics of $CD8^+$ T cells of the orally tolerant mice. PP $CD8^+$ T cells from the tolerant mice to the peptide neither proliferated nor produced cytokine mRNA upon *in vitro* stimulation with the peptide (data not shown). T cell clones from mesenteric lymph nodes of mice orally tolerized to MBP were reported to produce TGF-β in addition to IL-4 and IL-10 and suppress experimental autoimmune encephalomyelitis (EAE) (9). In contrast, $CD8^+$ T cells have been generated during oral tolerance in the Lewis rat, which triggers suppression of EAE (8). Therefore, it appears reasonable to consider that PP $CD4^+$ T cells producing IFN-γ, IL-2, IL-4, and TGF-β may be another T-helper cell subset with both mucosal T-helper function and down regulatory properties for systemic immune responses. Subpopulations of PP $CD4^+$ T cells remains to be elucidated. For this purpose, generation of regulatory $CD4^+$ T cell clones associated with oral tolerance are currently underway in our laboratories.

Our findings are in contrast to those of Clements et al. (2) suggesting that LT prevents the induction of tolerance to co-administered protein antigen, ovalbumin (OVA), and does not induce tolerance against LT itself as demonstrated by the presence of significant serum IgG and mucosal IgA anti-LT antibodies in immunized mice. Possible explanation for this discrepancy is that the forms of immunogen (20-mer peptide of LT-B versus LT holotoxin) may exhibit different effects on the antigen-presenting cells (APC), which could affect the outcome of the systemic antibody responses regulated by T cells.

Of significance is the finding that LT-B-specific IEL can rescue antigen-specific antibody responses from the influence of systemic unresponsiveness. Further, it is of interest that the IEL spontaneously produced both Th1-type and Th2-type cytokine mRNA (data not shown). IL-4, IL-5, and IL-6 are well recognized as the key cytokines for terminal differentiation of precursor B cells into plasma cells (15). In addition, IFN-γ upregulates the expression of B7/BB1 (CD80) on the APC (16) which delivers CD28-mediated co-stimulatory signal and prevents induction of anergy in T cell clones (17), suggesting that LT-B-specific IEL could abrogate SP $CD4^+$ T cell anergy. Taking these results into consideration, we propose that a clonal anergy or functional inactivation of the peptide-specific SP $CD4^+$ T cells is responsible for systemic unresponsiveness via the action of mucosally-derived $CD4^+$ T cells producing IFN-γ, IL-2, and TGF-β.

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