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Mucosal Immune Response and Adjuvant Activity of Genetically Fused *Escherichia coli* Heat-Labile Toxin B Subunit

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Abstract Although the *E. coli* heat-labile enterotoxin B subunit (LTB) is known to be a potent mucosal adjuvant towards co-administrated unrelated antigens and immunoregulator in T-helper 1-type-mediated autoimmune diseases, a more efficient and useful LTB is still required for prospective vaccine adjuvants. To determine whether a novel chimeric LTB subunit would produce an enhanced mucosal adjuvant activity and immune response, a number of LTB subunits were genetically fused with chimeric proteins using the epitope genes of the envelope glycoprotein E2 (gp51-54) from the classical swine fever virus (CSFV). It was found that the total serum immunoglobulin (Ig) levels of BALB/c mice orally immunized with chimeric proteins containing an N-terminal linked LTB subunit (LE1, LE2, and LE3) were higher than those of mice immunized with LTB, E2 epitope, and chimeric proteins that contained a C-terminal linked LTB subunit. In particular, immunization with LE1 markedly increased both the total serum Ig and fecal IgA level compared to immunization with LTB or the E2 epitope. Accordingly, the current results demonstrated that the LTB subunit in a chimeric protein exhibited a strong mucosal adjuvant effect as a carrier molecule, while the chimeric protein containing the LTB subunit stimulated the mucosal immune system by mediating the induction of antigen-specific serum Ig and mucosal IgA. Consequently, an LE1-mediated mucosal response may contribute to the development of effective antidiarrhea vaccine adjuvants.

Key words: Mucosal immune response, *Escherichia coli* heat-labile toxin B subunit, chimeric antigen, adjuvants, vaccine

The *E. coli* heat-labile enterotoxin (LT) and closely related *Vibrio cholerae* cholera toxin (CT) are both known as potent mucosal immunogens and immunoadjuvants, and have been demonstrated to enhance mucosal IgA as well as systemic antibody responses against co-administered or coupled antigens [21]. Although both toxins function as a mucosal adjuvant, the use of either toxin as an adjuvant of a mucosal vaccine for human use is not feasible owing to its toxicity. One approach being explored to resolve this problem is to use the nontoxic B subunits (LTB or CTB) that lack enzymatic activity [1] or B subunits coupled chemically or using gene fusion technology to vaccines [2, 10, 20, 28].

Meanwhile, another approach is the use of genetically detoxified derivatives of LTB or CTB. The genetic fusion of epitopes to LTB has been successful in some cases, but the genetic coupling of heterogeneous epitopes can also interfere with the structure, secretion, G_{M1}-binding, and immunogenicity of the LTB fusion proteins [2, 19]. Also, there are certain limitations to the size and type of antigen that can be attached to LTB by genetic fusion.

In the current study, it is assumed that some of the adjuvant properties of LTB can be explained by the increased uptake of LTB-coupled antigens across G_{M1}-expressing epithelial cells lining mucosal tracts. As such, the internalization of exogenous antigens has led to the suggestion that LTB can act as a carrier molecule for the direct delivery of antigens into mucus-associated lymphoid tissues (MALT). LTB and CTB also enhance the amount of antigen delivered to MALT inductive sites and subsequent stimulation of antigen specific B- and T-lymphocytes [11]. In general, LTB promotes both T-helper 1 (Th1) and 2 (Th2) cytokine responses [12], as well as the secretion of serum IgG1, IgG2a, IgG2b, and mucosal IgA. Furthermore, LTB can also modulate mucosal immune responses, such as lymphocyte apoptosis [25] and the expression of B-cell activation molecules [13].

The development of LTB as a vaccine has already been attempted for mucosal pathogens, including the classical

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swine fever virus (CSFV). CSF is a severe viral disease that attacks swine, characterized by fever, leukopenia, hemorrhages, and abortion, and results in high mortality. The causative agent, CSFV, belongs to the Pestivirus genus of the *Flaviviridae* family [27]. Pestiviruses are small, enveloped, positive-stranded RNA viruses and the genome, varying in length from 9.5 to 12.5 kb, contains a single large open reading frame [24]. The envelope glycoprotein E2 is the most immunogenic protein in CSFV. Thus, vaccines based on E2 can protect swine from CSF and induce high titers of neutralizing antibodies [6, 29], plus, no Abs directed against E^{rns} is induced, making these recombinant E2 vaccines potential CSF marker vaccines.

In a proposed model of the antigenic structure of E2, epitopes of neutralizing monoclonal antibodies have been mapped on two independently formed antigenic units, A/D and B/C [15], and vaccination with E2 containing one structural antigenic unit has been found to protect pigs from a lethal homologous CSFV challenge [16].

Accordingly, in the present study, the LTB subunit gene, E2 epitope, and chimeric genes were subcloned into an pET28a vector, expressed in BL21 (DE3), and the recombinant proteins were purified by nickel chelating affinity chromatography. Thereafter, mice were orally immunized with the purified LTB subunit antigen, E2 epitope antigens, and chimeric antigens and the antibody responses in the serum and fecal solution were assayed by an enzyme-linked immunosorbent assay (ELISA). As a result, it was demonstrated that the LTB subunit acted as a mucosal adjuvant in the genetically fused chimeric antigens using the epitope genes of the envelope glycoprotein E2 from CSFV. Consequently, the LTB subunit was found to induce mucosal and systemic immune responses in the gut.

MATERIALS AND METHODS

Virus, *E. coli*, and Mice

The vaccine strain of the CSF virus, strain ROM, was kindly provided by the ChoongAng animal disease laboratory (Daejeon, Korea), while the enterotoxigenic *E. coli* strain H10407 was purchased from the American type culture collection (ATCC, Manassas, VA, U.S.A.). Female BALB/c mice, at 7 weeks of age, were purchased from a commercial vendor (Samtako, Osan, Korea).

Cloning of LTB and E2 Gene

The genomic DNA was purified from *E. coli* H10407 using a Wizard genomic DNA extraction kit according to the manufacturer's manual (Promega, Madison, WI, U.S.A.). A fragment of the LTB gene was then amplified by a PCR reaction using an LTB1 primer (5'-TGG AAG CTT GCT CCC CAG ACT ATT ACA GA-3') and LTB2 primer (5'-TAC TGC AGT TTT TCA TAC TGA TTG-3') under the following conditions: 30 sec at 95°C, 60 sec at 45°C, and 90 sec at 72°C for 45 cycles. Next, the amplified LTB fragment was subcloned into the *Hin*dIII and *Pst*I sites of a pBluescript II SK(-) vector. The viral RNA of CSFV was purified using NucleoSpin VIRUS L (Macherey-Nagel GmBH, Wehingen, Germany), then 1 μg of the RNA was reverse transcribed into cDNA using a first strand synthesis kit (Roche, Germany). The E2 cDNA fragment of CSFV was amplified by a PCR reaction using an E2S1 primer (5-GTC GGA TCC CTA GCC TGC AAG GAA GAT-3') and E2AS1 primer (5'-TAC TGC AGA AAT ATA CTA CGA CCT CGA TGT-3') under the same conditions as above. The amplified E2 cDNA fragment was then subcloned into the pCRII-TOPO cloning vector (Invitrogen, U.S.A.), and the nucleotide sequences of these fragments analyzed using an ABI 377 auto sequencer (Applied BioSystem, Foster City, CA, U.S.A.).

Construction of LTB, E2 Epitope Genes, and Chimeric Genes

The primers, as shown in Table1, were designed with an additional termination codon for successful expression, and used for the PCR reaction performed with the LTB clone and E2 clone. The PCR conditions were 30 sec at 95°C, 60 sec at 52°C, and 90 sec at 72°C for 45 cycles. The amplified cDNA fragments were then subcloned into the *Bam*HI and *Hin*dII sites of a pBluescript II SK(-) vector (Fig. 1). To transfer the LTB, E2 epitope genes, and chimeric genes to an pET28a vector, the cDNA fragments were isolated by *Bam*HI and *Xho*I treatment of the clones, and subcloned into the pBluescript II SK(-), then subcloned to the *Bam*HI and *Xho*I sites in the pET28a vector.

Expression and Purification of Antigens

The genes encoding the LTB, E2 epitopes, and chimeric antigens were expressed in *E. coli* strain BL21 (DE3).

Table 1. Primer sequences for heat-labile enterotoxin B subunit and epitope genes from CSFV glycoprotein E2. The underline indicates the restriction enzyme cutting sites (GGATCC: *Bam*HI; AAGCTT: *Hin*dIII; CAGCTG: *Pvu*II), while the bold indicates the termination codon.

Primer	Sequence
E588s1	5'-GTC GGA TCC CTA GCC TGC AAG GAA GAT-3'
E588r1	5'-ATA AAG CTT CGC GTA GAC CAC TGG TTC ACC-3'
E210r1	5'-GCG AAG CTT CTT ATG CAA TGA TGC CAA AT-3'
E351s1	5'-TAC GGA TCC GAG CTC CTG TTC GAC GGG-3'
E588s2	5'-ACC AAG CTT CTA GCC TGC AAG GAA GAT-3'
E588r2	5'-ATA CAG CTG **CTA** CGC GTA GAC CAC TGG TTC ACC-3'
E210r2	5'-ATA CAG CTG **CTA** CTT ATG CAA TGA TGC CAA AT-3'
LTBs1	5'-TGG AAG CTT GCT CCC CAG ACT ATT ACA GA-3'
LTBr1	5'-GGC CAG CTG **CTA** GTT TTT CAT ACT GAT-3'
LTBr2	5'-GTA AAG CTT GTT TTT CAT ACT GAT TG-3'

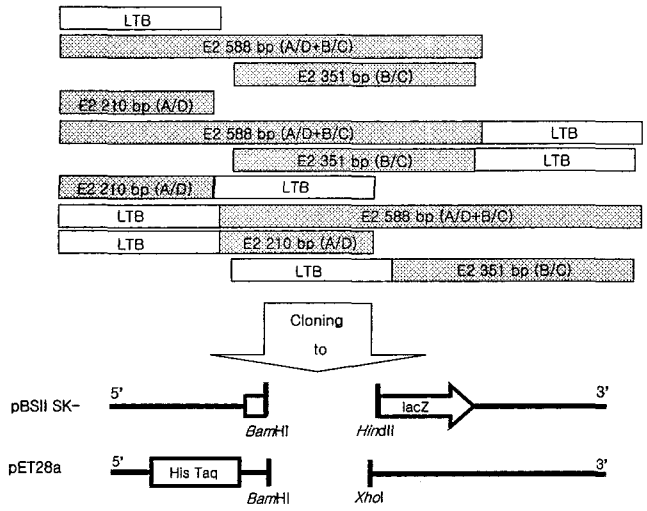

Fig. 1. Strategy used to subclone LTB subunit, E2 epitopes, and chimeric genes into pBluescript II SK(-) and pET28a plasmids. A 312 bp of the LTB subunit gene, 588 bp of the E2 epitope gene containing the A/D and B/C regions, 351 bp of the E2 epitope gene containing the B/C region, and 210 bp of the E2 epitope gene containing the A/D region were cloned into the *Bam*HI and *Hind*II sites of pBluescript II SK(-) and *Bam*HI and *Xho*I sites of pET28a. In the chimeric genes, the LTB subunit was cloned to the 5'-end and 3'-end flanked regions of the E2 epitope genes.

Ehrlenmeyer flasks containing 200 ml of Luria-Bertani (LB) broth supplemented with kanamycin were inoculated with 1% (vol/vol) inoculum from an overnight starter culture of strains carrying the appropriate plasmid. The cultures were grown with shaking (250 rpm) at 37°C overnight. Expression was induced by the addition of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 1 mM, then the cultures were incubated for a further 6 h before the cells were harvested by centrifugation [14]. As all the recombinant proteins lacked a signal peptide, they were expressed as cytoplasmic proteins and accumulated as inclusion bodies.

The cells were then resuspended in an appropriate volume of lysis solution (6 M guanidine-HCl, 20 mM phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4) and incubated at room temperature for 1 h. The resulting suspension was centrifuged at 4,000 rpm for 30 min and the supernatant was taken. The recombinant proteins were then purified using a HisTrap kit according to the manufacturer's manual (Amersham, Piscataway, NJ, U.S.A.). To maximize the assembly, the urea was gradually removed by dialysis. Meanwhile, the precipitates were removed by centrifugation and the protein solution was concentrated using an Ultrafree-CL centrifugal filter unit (Millipore, Bedford, MA, U.S.A.). Finally, the purified proteins were removed using Detoxi-Gel columns (Pierce, Rockford, IL, U.S.A.) and contained ≤50 endotoxin units per mg of proteins, as determined by a *Limulus* amoebocyte lysate assay (Biowhitteker, Walkerville, MO, U.S.A.).

Western Blot Analysis

SDS-PAGE was performed according to the method of Laemmli [8]. Appropriate amount of proteins were denatured by heating for 10 min in boiling water and loaded into each line of 13% SDS polyacrylamide gel. After separation, the proteins were transferred electrophoretically onto a nitrocellulose membrane (S&S, Keene, NH, U.S.A.) using a semi-dry trans-blotter (BioRad, Hercules, CA, U.S.A.). The membrane was blocked in a blocking solution (5% nonfat dry milk/0.2% Tween-20 in PBS) for 1 h at room temperature, then the tetra-His antibody (Qiagen, Hilden, Germany) was added to the blocking solution at a concentration of 1:2,000 and incubated for 1 h at room temperature. Any bound antibodies were detected using a 1:2,000 dilution of horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Pierce), with washes in PBS-T (0.2% Tween-20 in PBS) after each step. The membranes were developed using an ECL kit (Amersham) [22].

Immunization and Sampling

The immunogenicity of the LTB, E2 epitope proteins, and chimeric proteins was tested based on the oral immunization of groups of three mice with 5 μg of each antigen. The proteins were resuspended in phosphate buffered saline (pH 7.4; PBS) and delivered in a volume of 200 μl. A control group was immunized with the same volume of PBS. The mice were immunized on days 1, 7, 13, and 19 and the immune responses monitored from blood samples taken on days 10, 16, 22, and terminally on day 25. The blood samples were removed from the orbital plexus of each mouse and incubated for 2 h at room temperature. The serum was then separated from the clotted blood cells by centrifugation at 13,000 rpm for 30 min at room temperature, then stored at -20°C until assayed for the titer determination, as explained below. The feces from the mice were taken on day 22 and finally on day 25. The feces samples were suspended in 500 μl of PBS and the fecal extracts collected by centrifugation at 13,000 rpm at 4°C and stored at -20°C until used.

ELISA

The titers of the total serum immunoglobulin (Ig) were determined by ELISA for each antigen orally administered to the mice. The 96-well microtiter plates were coated with 2.5 μg of anti-mouse Ig (G+A+M) in PBS and incubated overnight at 4°C. The plates were then washed three times with tap water and blocked for 1 h at room temperature with 1% bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) in PBS. The serum from each mouse was tested using serial dilutions that were added to the washed plates and incubated for 1 h at room temperature. The plates were then washed, as described above, and incubated with a 1:1,000 dilution of HRP-conjugated goat anti-mouse

Ig (H+L) (BioTrend, Köln, Germany) for 1 h at room temperature. The bound antibodies were visualized by adding an *o*-phenylenediamine substrate, and the color development stopped by adding 10% H_2SO_4. The absorbancies were read at 490 nm using a Ceres 900HDi instrument (Biotek, Lafayette, IN, U.S.A.). Ig subclass and fecal IgA analyses were performed using an ImmunoPure Monoclonal Antibody Isotyping Kit according to the manufacturer's manual (Pierce), and the serum and fecal extracts from each mouse tested using four-fold serial dilutions.

RESULTS

Construction of LTB, E2 Epitope, and Chimeric Genes

The PCR was performed using the purified genomic DNA of *E. coli* strain H10407, and 320 bp of an amplified fragment was subcloned into pBlusccript II SK(-). The results of the restriction enzyme analysis and PCR sequencing analysis revealed that the subcloned fragment was 100% identical to the nucleotide sequences of the LTB (Fig. 2a), and this clone was named pL1. The E2 cDNA was amplified by a RT-PCR and subcloned into a pCRII-TOPO vector. In this case, the results of the restriction enzyme analysis detected about 1.2 kb of the predicted E2 cDNA fragment (Fig. 2b), and the nucleotide sequences of this fragment (pE2) exhibited a 97-99% homology with the E2 cDNA sequences of other virulent strains (Alfort [18] and ALD [7] strain), confirming that this fragment was the E2 cDNA of the ROM strain.

For the construction and expression of the chimeric genes, PCRs were performed with primers that contained a stop codon and the appropriate restriction enzyme sites. As a result, one set of fragments that contained a stop codon and another set that did not were amplified and these fragments subcloned into pBluescript II SK(-). The clones for the A/D+B/C epitope, A/D epitope, B/C epitope, and LTB subunit gene that contained a stop codon were named pE11, pE12, pE13, and pL3, respectively, while the clones that did not contain a stop were named pE5, pE6, pE7, and pL2, respectively. Six types of chimeric genes were constructed and subcloned into pBluscript II SK(-), in which the LTB were linked to a 5' flanked region of the three E2 epitope genes (pEL1, pEL2, and pEL3) and a 3' flanked region of the three E2 epitope genes (pLE1, pLE2, and pLE3) (Fig. 2c). Expressible fragments of the LTB subunits, E2 epitopes, and chimeric genes were isolated from the pL3, pE11, pE12, pE13, pEL1, pEL2, pEL3, pLE1, pLE2, and pLE3 plasmids and subcloned into pET28a, an *E. coli* expression vector (Fig. 2d).

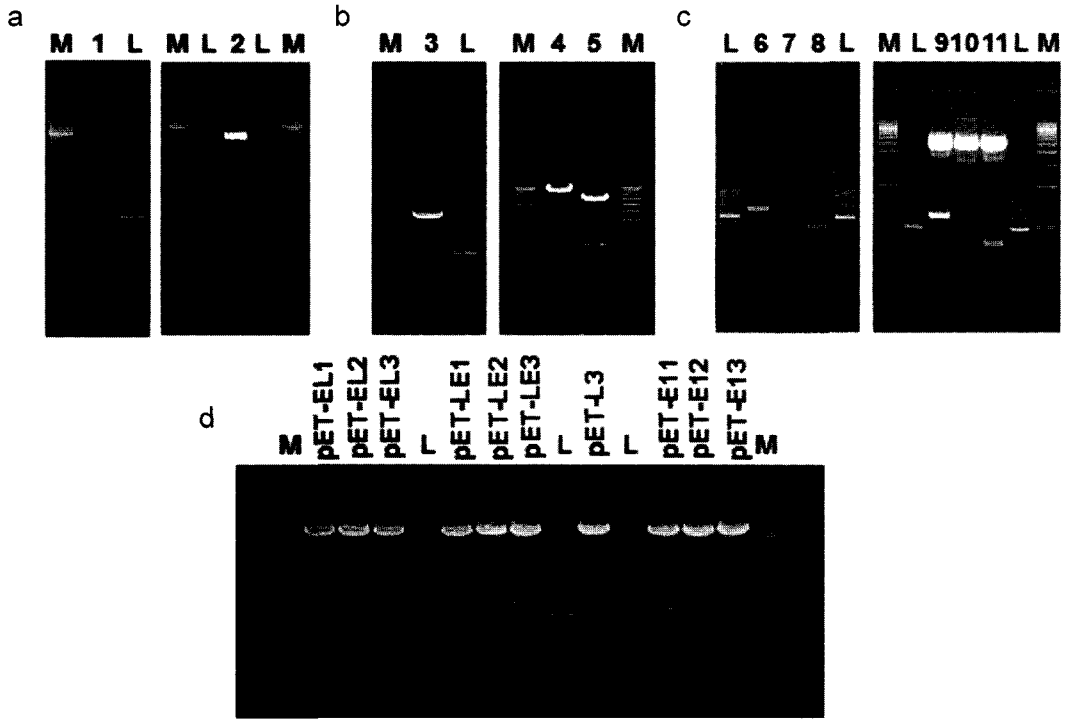

Fig. 2. Cloning results of PCR amplified LTB (a), E2 epitopes (b), and chimeric gene fragments (c) into pBluescript II SK(-) (a, c), pCRII-Topo (b), and pET28a (d).

M, 500 bp ladder; L, 100 bp ladder; Lane 1, LTB fragment; Lane 2, *Bam*HI/*Xho*I cut of pL1; Lane 3, E2 fragment; Lane 4, *Bam*HI single cut of pE2; Lane 5, *Eco*RI cut of pE2; Lane 6, A/D+B/C epitope fragment; Lane 7, B/C epitope fragment; Lane 8, A/D epitope fragment; Lane 9, *Bam*HI/*Xho*I cut of pE5; Lane 10, *Bam*HI/*Xho*I cut of pE6; Lane 11, *Bam*HI/*Xho*I cut of pE11.

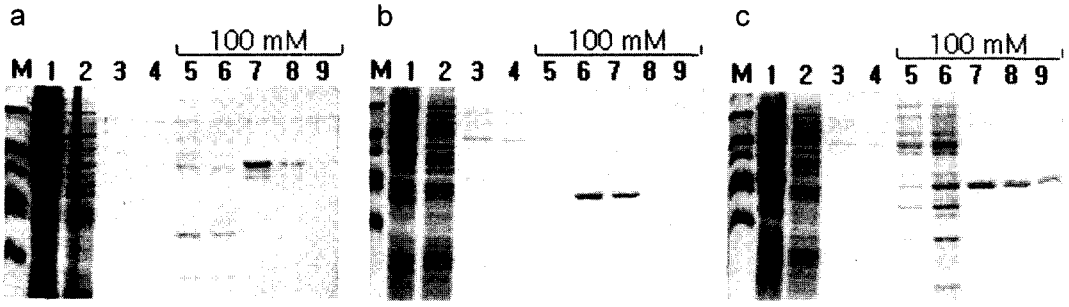

Fig. 3. Electrophoresis results for affinity purified LE1 (a), LE2 (b), and LE3 protein (c).
M, protein size marker; Lane 1, Lysate; Lane 2, Flow through; Lane 3, Wash 1; Lane 4, Wash 2; Lane 5, Elution 1 (100 mM imidazole); Lane 6, Elution 2 (100 mM imidazole); Lane 7, Elution 3 (100 mM imidazole); Lane 8, Elution 4 (100 mM imidazole); Lane 9, Elution 5 (100 mM imidazole).

Expression and Purification of LTB, E2 Epitopes, and Chimeric Proteins

The LTB subunit, E2 epitopes, and chimeric genes subcloned into pET28a were expressed in BL21 (DE3) by IPTG induction. Each clone exhibited different expression patterns and amounts of proteins. According to the SDS-PAGE results, all the recombinant proteins were successfully expressed with predicted molecular weights, although some proteins had a low expression level that was not detected by SDS-PAGE. To identify these proteins, a Western blot analysis was also performed with tetra-His antibodies that were able to detect accurate bands (data not shown).

The recombinant proteins were purified using nickel chelating affinity chromatography (Fig. 3). In the experiment, 100 mM of imidazole was identified as the optimal concentration for eluting the protein solutions, therefore, these conditions were used to elute the proteins (data not shown). As the purified recombinant proteins were dissolved in 8 M urea, the proteins were dialyzed with PBS, then concentrated to 100 μg per ml.

Antibody Responses to LTB, E2 Epitopes, and Chimeric Proteins

The relative immunogenicity of the purified recombinant proteins was examined based on the oral immunization of mice. Groups of three mice were orally immunized without anesthesia with 5 μg of each protein. The serum was separated from blood taken 3 days after the final immunization and the antibody responses determined by ELISA.

The total serum Ig level relative to the chimeric proteins that contained the LTB subunit fused to the N-terminal end of the E2 epitopes (LE1, LE2, and LE3) increased compared with the level relative to the LTB and E2 epitopes. However, the chimeric proteins that contained the LTB subunit fused to the C-terminal end of the E2 epitopes (EL1, EL2, and EL3) exhibited the same titer levels as the serum from the LTB and E2 epitope immunized mice (Fig. 4).

To investigate what kind of recombinant proteins stimulated mucosal immune responses, the secretary (S)-IgA titer from the fecal extracts was observed. The S-IgA titers for the LE1, LE2, and LE3 proteins that contained the N-terminal LTB-linked E2 epitope proteins were 2- to 3-fold higher than those for the LTB and E2 epitope proteins (Fig. 4), suggesting that the direction of the LTB subunit in these chimeric proteins was very important for enhancing the G_{M1} binding ability, while the N-terminal linked LTB subunit in the chimeric proteins functioned as a mucosal adjuvant.

Adjuvant Effects of LTB in Chimeric Protein

The total serum Ig and fecal S-IgA responses revealed only LE1, where the LTB was fused to the N-terminal end of

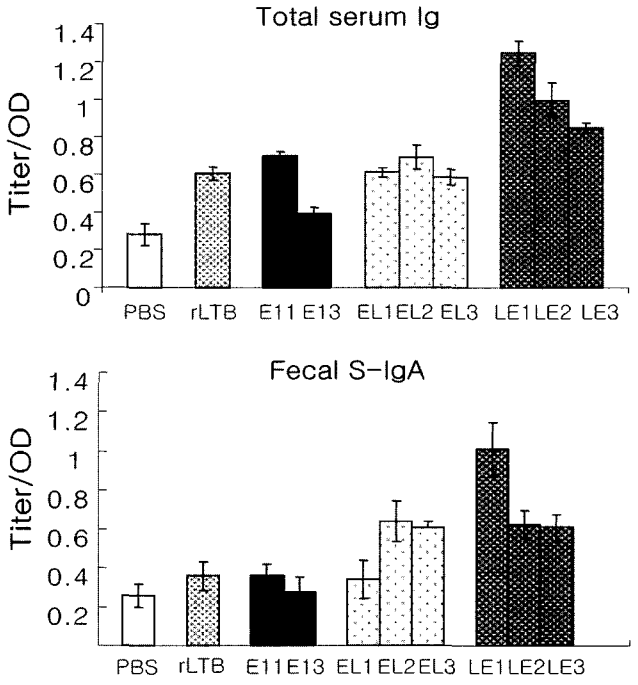

Fig. 4. Kinetics of total serum antibody and fecal S-IgA responses to orally immunized recombinant LTB subunit, E2 epitopes, and chimeric proteins.
The mice were immunized with 5 μg of the recombinant LTB subunit, E2 epitopes, and chimeric proteins. Serum samples and fecal extracts were taken 3 days after the final immunization. The error bar indicates the standard deviations of the mean responses (n=3 mice per group).

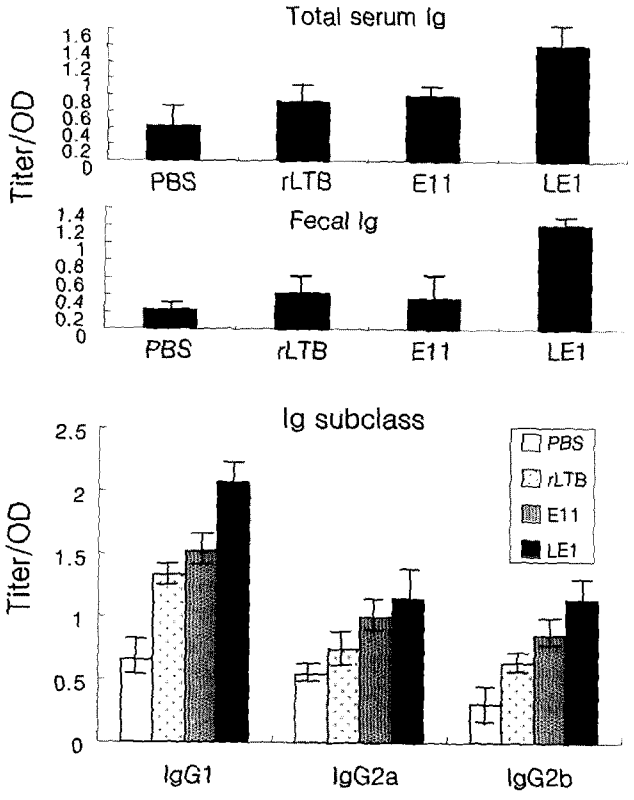

Fig. 5. Mucosal adjuvant effects of N-terminal linked LTB in chimeric protein and Ig subclass analysis of chimeric protein. The mice were immunized with 5 μg of the recombinant LTB subunit, E2 epitopes, and chimeric proteins. Serum and fecal samples were taken 3 and 7 days after the final immunization. The error bar indicates the standard deviations of the mean responses (n=3 mice per group).

E11 stimulated 2- to 5-fold more than LTB and E11 (Fig. 5), indicating that the antibody responses to the E2 epitope were increased by an adjuvant effect of the LTB subunit. The nature of the immune response to the LTB subunit in the chimeric protein LE1 as an adjuvant was determined by the analysis of the Ig subclass distribution of the serum antibody responses in comparison with a negative control, LTB and E11 (Fig. 5). The immunized LE1 protein triggered an immune response dominated by the presence of the Th-2-associated antibody subclass, IgG1. However, IgG2a, a Th-1-associated antibody was also triggered, suggesting that the LTB containing the chimeric protein LE1 induced a balanced Th1/Th2 immune response and modulated the cell-mediated immunity and humoral immunity.

DISCUSSION

The results presented in this paper demonstrated that LTB in a chimeric protein functions as a mucosal adjuvant. In particular, the chimeric protein LE1 with an N-terminal linked LTB subunit stimulated serum Ig and fecal S-IgA responses. Thus, it is postulated that the ability of LTB to act as an adjuvant in this chimeric protein resulted from the receptor specificity and stability.

The LTB subunit is known to bind to a wider range of receptors, including surface gangliosides G_{M1}, G_{D1b} asaio-G_{M1}, lactosylceramide, and certain galactoproteins [3, 5]. The role of these alternative receptors for the LTB subunit in mediating its effects on the immune system has not yet been explored. The LTB subunit is also stable at low pH, as a pentameric structure and remains stable down to pH 2.0 [17]. The pentameric formation of LTB in the chimeric proteins was not investigated in the current study. However, the results of the antibody responses to the chimeric proteins that contained the N-terminal linked LTB subunit compared to that which contained the C-terminal linked LTB subunit indicated that the LTB subunits did probably form pentamers, and the direction of the LTB subunit in the chimeric proteins was very important for the formation of a pentameric structure.

Interestingly, other recent reports have shown that LTB does not exhibit an adjuvant effect when orally immunized with unrelated antigens [4, 26], which contradicts the current data, where the LTB subunit included in the chimeric protein did act as a mucosal adjuvant.

An analysis of the IgG subclass responses revealed that the chimeric protein LE1 stimulated both IgG1 and IgG2a responses, yet the IgG1 level was relatively higher. However, in the case of LTB and CT, the serum antibody responses were dominated by the presence of IgG1, with relatively low levels of IgG2a. This interesting observation indicates that whereas LTB and CT stimulate predominantly Th2 response [9], LE1 produced more balanced Th1 and Th2 responses, like LT [23]. This suggestion was also supported by data from a cytokine analysis, where Peyer's patch cells from the orally immunized mice produced high levels of interleukin-4, interferon-γ, and transforming growth factor-β (data not shown). As such, these results indicate that the LTB in the chimeric protein LE1 or the chimeric protein induce $CD4^+$ T-cell-mediated humoral immunity and $CD8^+$ T-cell-mediated immune responses.

Accordingly, the current study highlighted the potential of LTB in a chimeric protein as an effective adjuvant capable of triggering mucosal and systemic immunity. Therefore, these findings should facilitate the development of mucosal vaccination strategies without the residual toxicity associated with the use of holotoxin.

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