approaches to mucosal immune therapy and mucosal vaccine design, eventually contributing to the improvement of public health.

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Production of a Recombinant Cholera Toxin B subunit-Insulin B chain Peptide Hybrid Protein by *Brevibacillus choshinensis* Expression System as a Nasal Vaccine Against Autoimmune Diabetes

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Abstract: Mucosally induced tolerance is an attractive strategy for preventing or reducing autoimmune diseases. Here, we produced a recombinant CTB fusion protein linked with autoantigen T cell epitope of insulin B chain peptide 9–23 (C19S) at levels up to 200 mg/L culture media in *Brevibacillus choshinensis* secretion-expression system. Receptor-competitive assay showed that the CTB-insulin peptide binds to GM1 receptor almost equivalent degree as the native form of CTB. Non-obese diabetes (NOD) mice that spontaneously develop an insulin-dependent diabetes were nasally immunized with CTB-insulin peptide (5 μg) for three times. The nasal treatment significantly reduced the development of insulin-dependent diabetes and peptide specific DTH responses after systemic immunization with the insulin peptide B 9–23(C19S) in CFA. Nasal administration of as high as 50 μg of the peptide alone demonstrated a similar level of the disease inhibition. In contrast, all mice given 5 μg of the insulin peptide alone or 5 μg of insulin peptide with 25 μg of the free form of CTB did not lead to the suppression of diabetes development and DTH responses. Because molecular weight of the insulin peptide is about one tenth of that of the CTB-insulin peptide, the results demonstrate that the recombinant hybrid of autoantigen and CTB increased its tolerogenic potential for nasal administration by up 100-fold on molar base of autoantigen peptide. Taken together, nasally-induced tolerance by administration of the recombinant *B.choshinensis*-derived hybrid protein of CTB and autoantigen T cell-epitope peptide could be useful mucosal immunetherapy for the control of T cell-mediated autoimmune diseases.

Keywords: cholera toxin b-subunit; insulin peptide; diabetes; autoimmune diseases; vaccine

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INTRODUCTION

Insulin-dependent type 1 diabetes mellitus (IDDM) is an autoimmune disorder in which the insulin-producing beta cells are specifically destroyed. IDDM currently affects an estimated of 0.4 percent of population in the world (Vyse and Todd, 1996). Insulin has saved the lives of millions of IDDM patients. Insulin preparation with new type of syringes has made it possible to improve diabetes management. Because careful diabetes control reduce the risk for all diabetes complications. Currently, however, none of treatment is available for the prevention or cure of IDDM (Tisch and McDevitt, 1996). In order to induce diseases remission, a treatment with immunosuppressive drugs such as steroid or cyclosporin has had some effects in preserving residual β-cell function in new onset patient (Elliott et al., 1981; Feutren and Mihatsch, 1992). However, most of that often lead to serious side effects. An alternative to the immunosuppressive treatment of IDDM is therapeutic induction of immune tolerance to autoantigen. Mucosal induced tolerance such as oral tolerance has been used in various autoimmune diseases in clinical studies to inhibit the undesired immune response against autoantigens (Chen et al., 1996; Wu and Weiner, 2003). Although there were no major side effects, it often required repeated feeding of large amounts of tolerogen over 1–2 years and was only partially effective in patients with autoimmune diseases. The treatment may be limited efficacy in pre-sensitized animals or patients with existing auto-imuune diseases (Zhang et al., 1991).

Promising studies indicated that autoantigen chemically conjugated to immunomodulator such as cholera toxin B-subunit (CTB), may be effective in both prophylaxis and

treatment of pre-existing autoimmune condition (Bergerot et al., 1997; Phipps et al., 2003). Nevertheless, chemical coupling may lead to the generation of a heterogeneous preparation or potential alteration of CTB-conjugates, which is not a suitable for clinical use. The aim of our study is to develop a safe and uniform recombinant CTB-autoantigen peptide for possible clinical use. We now report that nasally induced tolerance by administration of a small dose of recombinant CTB-insulin peptide B-9-23 fusion protein protects female NOD mice against spontaneous autoimmune diabetes. It is demonstrated that coupling of the insulin peptide to CTB was increased its tolerogenic potential after nasal administration by up 100-fold on molar base of the peptide. These results have important implication for the development of T cell epitope-based mucosal immunotherapy of IDDM.

MATERIALS AND METHODS

Materials

Brevibacillus choshinensis 47 K is a mutant of the strain 47 isolated by (Udaka, 1976; Yamagata et al., 1989). Recombinant CTB was produced by the method of Ichikawa et al. (1993) and purified by using immobilized galactose (Pierce Chemical, Rockford, IL) (Uesaka et al., 1994). A peptide used in the control experiment, S-H-L-V-E-A-L-Y-L-V-S-G-E-R-G [insulin peptide B 9–23(C19S)] (Daniel and Wegmann, 1996), was chemically synthesized on a Applied Biosystems 430A peptide synthesizer (Foster City, CA). The peptide was more than 95% pure as determined by HPLC. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc. (Beverly, MA).

Construction of Expression Vectors

A CTB/pNU212 expression vector containing *CTB* coding gene from *Vibrio cholerae* 569B and the promoter and signal peptide-encoding region of cell-wall protein gene of *B. choshinensis*, was prepared according the method described previously (Ichikawa et al., 1993). The open reading frame of a gene encoding CTB conjugated with the insulin B peptide was amplified by PCR from the CTB/pNU212 using the following primers.

For sense: 5′-CTCCCATGGCTTTCGCTACACCTCAA-AATATTACTG-3′; for antisense: 5′-CGTCGAAGCTTA-GCCTCGTTCCCCGGACACTAGGTAGAGAGCTTCCA-CCAGGTGTGAGCCTGGACCATTTGCCATACTAA-3′. The resulting PCR products were cloned into a pT7Blue vector (Novagen, Inc., Madison, WI). These plasmids were transformed into *E.coli* NovaBlue competent cells (Novagen). Bacteria were grown at 37°C for 1 h and plated on LB plates containing 25 mg/mL of ampicilin, 35 mL of 25 mg/mL X-gal, and 20 mL of 100 mM IPTG. Several strains carrying CTB-insulin-B-peptide gene were grown at 37°C for 16 h and plasmids were purified by the alkaline-extraction method (Birnboim, 1983). After digestion with NcoI I and Hind III, plasmids were fractionated on an agarose gel and then the 378-base pairs of CTB-insuln-B-peptide gene fragment was purified by using GENE-CLEAN (Bio101, Vista, CA). After ligation, the plasmid DNA containing the CTB-insulin-B-peptide gene (*CTB*-inp/pNU212) was introduced into *B.choshinensis* 47 K by the electroporation method (Okamoto et al., 1997).

Expression and Purification of CTB-Insulin B Peptide Hybrid Protein

B.choshinensis 47 K carrying *CTB*-inp/pNu212 gene was grown for 3 days at 30°C in S2U media containing 40 g of Soytone (Difco, Detroit, MI), 10 g of yeast extract (Difco), 30 g of glucose (Sigma Chemical Co., St. Louis, MO), 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of $MgCl_2 \cdot 7H_2O$, and 0.1 g of Uracil (Sigma) per liter (pH 7.0). The culture supernatant (1 L) were concentrated fivefold with an ultrafiltration device (Amicon: Beverly, MA) through a 10,000 molecular weight cut-off filter. After centrifugation (20 min, 20,000*g*), the supernatant was precipitated with ammonium sulfate at 80% saturation, followed by dialyzed against 50 mM Tris-HCl buffer (pH 7.4) containing 0.2 M NaCl, 3 mM NaN_3, 1 mM EDTA (TEAN buffer). The dialysate was applied to a DEAE-Sepharose (Pharmacia Biotech, Alameda, CA) column (5 × 30 cm) equilibrated with TEAN buffer. The through-fraction was pooled and concentrated using the Amicon and then was applied to a galactose-immobilized gel (Pierce Chemical; Uesaka et al. (1994)) column (2 × 15 cm) equilibrated with TEAN buffer. After washing with TEAN buffer, CTB-insulin peptide fusion protein was eluted with 0.3 M galactose in TEAN buffer. The active fraction was pooled, concentrated by Amicon and then applied to a Sephadex G-100 (Pharmacia Biotech) column (2 × 95 cm) equilibrated with PBS, pH 7.4. The protein concentration was determined, based on the result of amino acid analysis using a Beckman 6300 amino-acid analyzer (Beckman Instruments, Fullerton, CA) after hydrolysis. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed under non-reducing conditions using the method described by Laemmli (1970). The amount of endotoxin was measured in the sample preparation with an Endospec-SP test (Seikagaku Co., Tokyo, Japan).

Amino Acid Sequence of CTB-Insulin B Peptide Hybrid Protein

Amino acid sequence analysis was carried out using the method of Byun et al. (2001). Briefly, purified CTB-insulin B peptide hybrid protein was digested with endopeptidase Lys-C (1:50 w/w, Wako Chemicals, Richmond, VA) at 37°C. Each digested sample was separated by reverse-phase HPLC on a C18 HPLC column (0.46 × 15 cm; Vydac, Hesperia, CA) using a 0.1% trifluoroacetate (Buffer A) –80% acetonitrile in 0.1% trifluoroacetate (Buffer B) gradient system. Each peak was collected, then analyzed by a protein sequencer 610A (Perkin Elmer/Applied Biosystems, Foster City, CA).

GM1 Receptor ELISA

GM1 receptor ELISA was carried out using the modified method of Dertzbaugh and Elson (1993). Briefly, wells of a polyvinyl microtiter plate were coated with 100 ng of GM1 ganglioside (Sigma). Wells were blocked with 1% bovine serum albumin in Tris-buffered saline, pH 7.5 (BSA–TBS). Each protein was adjusted to an equimolar concentration and then serially diluted twofold in BSA–TBS. Each dilution was mixed with an equal volume of biotinylated CTB (List Biologics, Campbell, CA) diluted to a concentration of 100 ng/0.1 mL. After incubation for 2 h at room temperature, the plate was washed and horseradish peroxidase conjugated streptavidin (Pierce Biotechnology, Rockford, IL) was added. The plate was incubated for 2 h at room temperature and, after washing, developed at room temperature with 100 µL of chromogenic substrate, 3,3′, 5,5′-tetramethylbenzidine with H_2O_2 (Moss, Pasadena, MD). Reactions were terminated by adding 50 µL of 0.5 M HCl.

Induction and Reduction of Diabetes

Female NOD mice purchased from the Jackson Laboratory (Bar Harbor, ME), spontaneous diabetes starts approximately 11 weeks of age and reached an incidence of 70% by 28 weeks in our colony. Diabetes was characterized by polydipsia, weight loss, glycosuria as assessed by urine Chemstrips (Bayer, Germany), and persistent hyperglycemia, which was determined with blood glucose levels using a Glucometere (Bayers, Germany) (Bergerot et al., 1997). Mice with a blood glucose level above 100 mg/dL were scored as diabetes (Daniel and Wegmann, 1996). To assess the reduction of diabetes, antigen-specific, mucosally induced tolerance was induced by nasal administration of 5 or 25 µg CTB-insulin B peptide hybrid protein in weeks 6, 7, and 8. Mice receiving 5 or 50 µg insulin B peptide alone or insulin B peptide (5 µg) with CTB (25 µg) were used as a control group. Mice were routinely monitored for diabetes by 40 weeks. The Cox-Mantel log rank test was used for statistical analysis of the data.

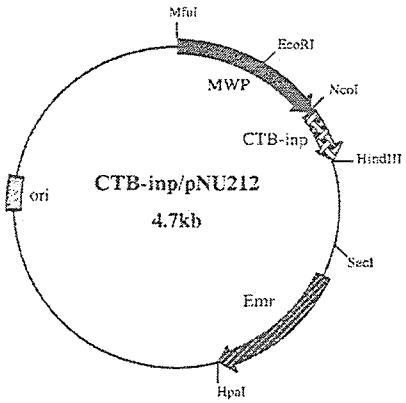
Measurement of DTH Responses

Female NOD mice were immunized with 30 µg of insulin B 9–23 (C19S) in CFA at base of the tail in 9 weeks after nasally immunized in weeks 6, 7, and 8 with 5 µg of the CTB-insulin B peptide hybrid protein, 5 or 50 µg of the insulin B peptide alone, or 5 µg of insulin B peptide together with 25 µg of CTB or non-treatment. In order to induce DTH responses, 1 week after the last systemic immunization, 20 µg of insulin B 9–23 (C19S) in PBS was subcutaneously injected into the right-hind footpad of the mice (Johnson et al., 1998). The left-hind footpad received PBS as a control. Footpad thickness was measured with a gauge before and 24 h after the injection, and the difference in footpad thickness between the right and left footpads was taken as the specific response. The Dunnett's test was used for statistical analysis of the data (Johnson et al., 1998).

RESULTS

Expression and Purification of CTB-Insulin B Peptide Hybrid Protein

The expression vector pNU212 containing CTB-insulin-B-peptide gene is shown in Figure 1. After introduction of



CTB-insulin B peptide(C19S)

CTB Linker Insulin B 9-23

AAT GGT CCA GGC TCA CAC CTG GTG GAA GCT CTC TAC CTA GTG TCC GGG GAA CGA GGC TAA GCT TCG ACG
Asn Gly Pro Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Ser Gly Glu Arg Gly *

Figure 1. Construction of CTB-insulin B 9–23 fusion protein expression-secretion vector CTB-inp/pNU212. The promoter and signal peptide of the *MWP* gene are represented by the hatched bar and the sequence coding for CTB-insulin B 9–23 (C19S) by the filled bar. Arrows indicate the direction of transcription. "Ori" signifies "origin of replication." The nucleotide and amino acid sequences around the linking site with a linker and insulin B 9–23 (C19S) of the fused gene are shown at the bottom.

CTB-inp/pNU212 into *B.choshinensis*, the clones producing CTB-insulin B peptide hybrid protein were identified in culture supernatants by SDS–PAGE followed by coomassie brilliant blue staining. A time course study showed that the rate of CTB insulin B peptide production increased for up to 3 days of incubation and remained constant afterward (data not shown). Finally, secretion level of CTB insulin B peptide could be achieved at up to approximately 250 mg/L. A highly purified CTB-insulin B peptide hybrid protein was obtained from a 3-day culture supernatant of the *B.choshinensis* by using ammonium sulfate precipitation, a DEAE-Sepharose chromatography, a galactose-immobilized affinity chromatography followed by gel filtration on a Sephadex G-100. Using this procedure, 200 mg of purified hybrid protein was obtained from 1-L culture supernatant. The purified CTB-insulin B peptide hybrid protein was more than 95% pure as determined by SDS–PAGE in reducing conditions (data not shown). The purified CTB-insulin B peptide hybrid protein and formerly purified recombinant CTB contained 2.9 and 5.1 pg of lipopolysaccharide (LPS) per 100 μg of protein, respectively.

Characterization of CTB-Insulin B Peptide Hybrid Protein

The SDS–PAGE analysis on non-reducing conditions of purified recombinant CTB and CTB-insulin B peptide hybrid protein revealed a pentamer formation (Fig. 2). The pentameric form of purified recombinant CTB and CTB-insulin B peptide hybrid protein migrated with a molecular size of 40 and 45 kDa, respectively. The N-terminal amino acid sequence of purified CTB-insulin B peptide hybrid protein, TPQNITDLCAEYHNTQI, was identical to that of native CTB (Mekalanos et al., 1983). It was also found a peptide including insulin B 9–23 (C19S) amino acid sequence after the C-terminal of CTB, TPHAIAAIS-MANGPGSHLVEALYLVSGERG, after lysyl endopeptidase digestion of pyridylethylated CTB-insulin B peptide hybrid protein.

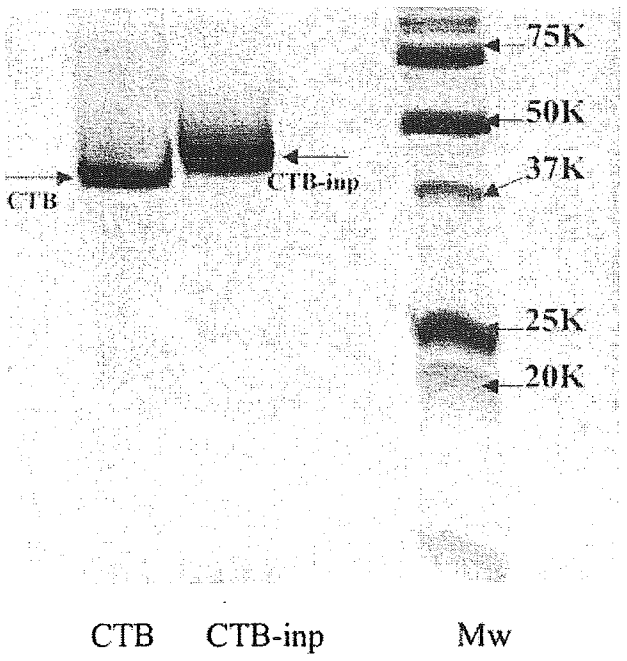


Figure 2. SDS–polyacrylamide gel electrophoresis for the CTB-insulin B 9–23 fusion protein purified CTB and CTB insulin B 9–23 (C19S) fusion protein (CTB-inp) were electrophoresed in 12% ployacrylaminde gel with 0.1% SDS under non-reducing condition, respectively. The molecular weight maker (Mw) is shown on the right side of the gel.

To verify whether the CTB-insulin B peptide hybrid protein binds to GM1, the affinity of the protein for GM1 was compared with that of native recombinant CTB by using a competitive ELISA. When increasing amounts of the CTB-insulin B peptide hybrid protein were mixed with a constant concentration of biotinylated CTB and then reacted with GM1, the CTB-insulin B peptide hybrid protein was found to bind to GM1 with an equivalent binding affinity as the native form of CTB (Fig. 3). For CTB to bind to GM1 receptors, it must form a pentameric structure composed of identical monomers (Hardy et al., 1988). These findings suggest that the *B.choshinensis*-derived recombinant hybrid protein of CTB-insulin B peptide have a native pentamer form of CTB linked with diabetes inducing insulin peptide B 9–23 (C19S).

Nasal Administration of CTB-Insulin Peptide Hybrid Protein Reduced the Development of Diabetes

The female NOD mice began to develop spontaneously diabetes about 11 weeks of age and reached an incidence of 70% by 30 weeks in the colony used. Diabetes was characterized by polydipsia, weight loss, glycosuria, and persistent hyperglycemia. As shown in Figure 4, when CTB-insulin B

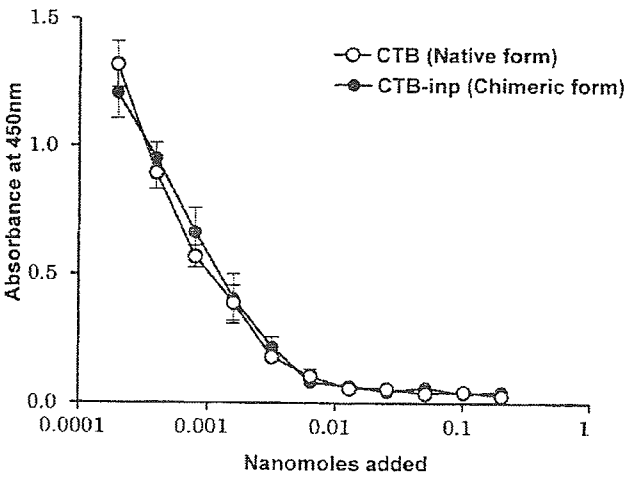


Figure 3. Comparison of the affinities of CTB-insulin B 9–23 fusion protein and the native form of CTB for the GM1 receptor by competive ELISA. Each protein is adjusted to an equimolar concentration. Serially diluted twofold concentrations of individual sample are then mixed with a fixed amount of biotinylated CTB and reacted with GM1 bound on the solid phase. Data are average of five measurements.

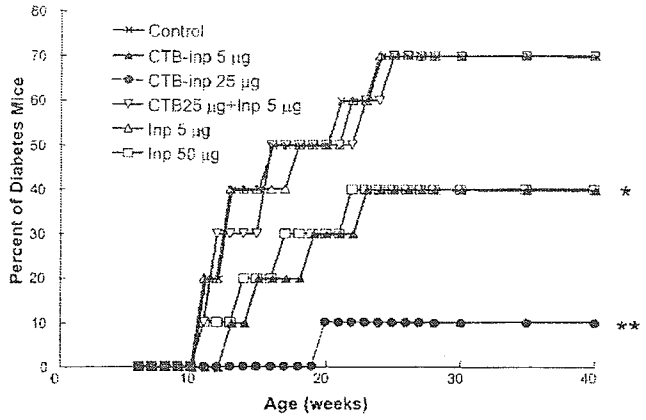


Figure 4. Suppression of spontaneous autoimmune diabetes development in NOD mice after nasal immunization with CTB-insulin B 9–23 fusion protein. The immunogens were nasally administered three times on 1 day in 6, 7, and 8 weeks. Mice (10 animals per group) were routinely monitored for diabetes by 40 weeks. Diabetes was characterized by polydipsia, weight loss, glycosuria as assessed by urine Chemstrips, and persistent hyperglycemia, which was determined with blood glucose levels using a Glucometere. Mice with a blood glucose level above 100 mg/dL were scored as diabetes. *: $P < 0.05$ Cox-Mantel log rank test. **: $P < 0.01$ Cox-Mantel log rank test.

peptide hybrid protein was administered via the nasal route, mice receiving three doses of 5 or of 25 μg of the fusion protein dose-dependently showed a reduction of percent of onset of diabetes. In addition, mice were nasally administered three times with 5 or 50 μg of insulin B 9–23 (C19S) peptide alone (Fig. 4). The nasal administration of 50 μg insulin B 9–23 (C19S) peptide alone show almost the same level reduction of onset of diabetes with that of 5 μg of the CTB-fusion protein. Because 5 μg of the CTB-insulin B peptide fusion protein contained only 0.6 μg of insulin B 9–23 (C19S) peptide from a ratio of their amino acid residues (15/121), the result demonstrate that coupling of autoantigen to CTB is increased its healing potential after nasal administration by up 100-fold on molar base of autoantigen peptide. For comparison purposes, mice were nasally administered three times with 5 μg of insulin B 9–23 (C19S) peptide mixed with 25 μg of the recombinant CTB. The control nasal treatment did not provide the inhibitory effects necessary for reduction of the percent of onset of diabetes (Fig. 4). The result also showed non-conjugated form of CTB did not enhance the induction of immune tolerance after oral insulin peptide administration. These results show that the CTB-insulin B peptide hybrid protein is one of the most effective molecules for the inhibition of the diabetes via the nasal route.

Nasal Administration of CTB-Insulin Peptide Hybrid Protein Reduced Insulin-Peptide Specific DTH Responses in the Diabetes Mice

Our next experiment was aimed at investigating whether nasally induced tolerance was established by insulin B 9–23 (C19S)-peptide specific cell-mediated immune responses because nasal immunization with small amount of the hybrid protein of CTB insulin B peptide led to the reduction of diabetes development. In order to this aim, the NOD mice were subcutaneously immunized with the insulin peptide together with CFA after nasal administration with CTB-insulin B peptide hybrid protein or insulin peptide alone or the insulin peptide plus CTB. The insulin B 9–23 (C19S) peptide-specific DTH responses were measured in 24 weeks after challenge with the insulin B 9–23 (C19S) peptide. As in Figure 5, DTH responses were reduced at almost the same level after nasal administrations of 5 μg CTB-insulin B peptide hybrid protein or 50 μg insulin B 9–23 (C19S) peptide alone subsequent to immunization with the insulin B peptide in CFA. On the other hand, the mice receiving nasal treatment with 5 μg the peptide, either alone or mixed with 25 μg CTB, did not lead to the reduction of peptide specific DTH responses (Fig. 5). These findings demonstrate that tolerance can be nasally induced with the use of hybrid protein of CTB and insulin B peptide.

DISCUSSION

IDDM results from immune-mediated destruction of β cells. Insulin is the only known islet autoantigen produced by β cells (Tisch and McDevitt, 1996). Thus, loss of tolerance to insulin is considered to direct affect β cells-specific destruction. Mucosally induced tolerance including both oral and nasal tolerance to autoantigen has been considered as an attractive strategy for preventing or reducing autoimmune diseases such as IDDM (Wu and Weiner, 2003). Although there were no major side effects, induction of oral tolerance requires repeated administration of large amount of autoantigen (Chen et al., 1996). It was shown that a small amount of oral CTB conjugated chemically to insulin prevented spontaneous autoimmune diabetes in NOD mice (Bergerot et al., 1997; Petersen et al., 2003). However, this chemical coupling procedures may affect the immunogenecity of the chemically modified antigen and may lead to the generation of a heterogeneous population of CTB conjugates (McGhee

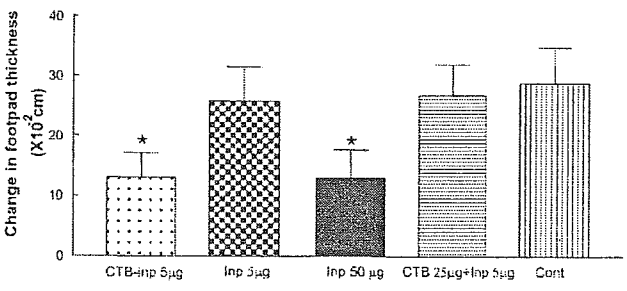


Figure 5. Nasally immunized CTB-insulin B 9–23 fusion protein inhibits insulin peptide-specific DTH responses. The immunogen were nasally administered three times on 1 day in 6, 7, and 8 weeks before systemic challenge for the induction of DTH. After 1 week, all nasally treated mice (10 animals per group) were given 20 μg of insulin B 9–23 (C19S) or saline subcutaneously in the right and left footpads, respectively. Footpad thickness was measured before and 24 h after challenge. The differences in footpad swelling between the two footpads were taken as DTH responses. *: $P < 0.05$ versus control group by Dunnett's test.

et al., 1992). In as much as NOD mouse is considered to be a good model of IDDM (Tisch and McDevitt, 1996), we used the NOD-mouse system to examine the effectiveness of chimeric molecule of CTB and autoantigen T cell epitope of insulin. Thus, we produced the hybrid protein of CTB and insulin B chain peptide 9–23, which is a dominant T cell epitope of the mouse (Daniel and Wegmann, 1996; Devendra et al., 2004), as an effective nasal vaccine against spontaneous autoimmune diabetes using *B.choshinensis* expression system to generate a safe and uniformed immunogen for clinical use.

The use of *B.choshinensis* as a recombinant hybrid protein expression system has many of advantages. First, the *B.choshinensis* system produces high levels of recombinant proteins in culture medium with little contamination of lipopolysaccharide (Inoue et al., 1997). When *B.choshinensis* 47 K is used as a host, CTB-insulin B peptide fusion protein that highly expressed and extracellularly secreted are stably maintained. In fact, 200 mg of the CTB fusion protein per liter could be recovered from the culture medium. In addition, the purified CTB-insulin B peptide fusion protein contained only 2.9 pg of LPS per 100 μg. The range LPS (e.g., less than 50 pg/100 μg) was been shown to have no biological effect on the immune system (Ultrich et al., 1991). Furthermore, sequence analysis confirmed the fusion protein has full sequence of CTB and insulin B peptide 9–23 (C19S). Second, the secreted recombinant protein produced in *B.choshinensis* has shown to form correctly folded structure with appropriate biological activity. *B.choshinensis* has the very low level of extracelular protease activity, which promotes stability of the secreted recombinant proteins (Shiroza et al., 2003). The *B.choshinensis* derived CTB-insulin B peptide fusion protein is secreted as pentamer (Fig. 2) and GM-1 receptor assay showed the fusion protein binds to GM-1 receptor an almost equivalent degree as the native form of CTB (Fig. 3). CTB pentamer functions as a tolerogen based on its affinity for cell surface receptor GM-1 gangliosides expressed by epithelium in mucosal inductive sites such as the gut-associated lymphoreticular tissues (GALT) (Frey et al., 1996). Third, *B.choshinensis* is considered a safe microorganism for production of recombinant proteins that will be administered to animals or humans because the bacteria are known to be harmless resident of soil (Udaka and Yamagata, 1993). These unique characteristics of the *B.choshinensis* recombinant protein expression system result in production of uniform CTB-insulin B peptide fusion protein.

In this study, we produced a recombinant CTB fusion protein linked with insulin B chain peptide 9–23 (C19S) to examine its inhibitory effect on the development of spontaneous autoimmune diabetes. When CTB-insulin peptide hybrid protein was administered via the nasal route, mice receiving three doses of 5 or of 25 μg of the fusion protein showed dose-dependently a reduction of percent of onset of diabetes (Fig. 4). The nasal administration of 50 μg insulin B peptide 9–23 (C19S) alone show almost the same level reduction of onset of diabetes with that of 5 μg of the CTB-fusion protein (Fig. 4). The recombinant hybrid of insulin peptide and CTB is effective at doses 100-fold less than the autoantigen peptide (Fig. 4). The pentametric structure of the CTB fusion protein not only facilitate GM1 ganglioside specific delivery and presentation of conjugated peptide to the GALT, it also increases the molar concentration of autoantigen peptide per molecule of CTB pentamer (Arakawa et al., 1998). The increase in antigen peptide concentration in GALT may reduce the requirement for high concentration of autoantigen synthetic peptide in CTB fusion protein.

In this study, the nasal administration of CTB-insulin B peptide hybrid protein was shown to induce a significant reduction of insulin B peptide-specific DTH responses (Fig. 5). In addition, the recombinant hybrid of insulin peptide and CTB also increased its tolerogenic potential for nasal administration by up 100-fold on molar base of autoantigen peptide (Fig. 5). Because DTH responses are thought to be regulated by $CD4^+$ Th1 cells (Stevens et al., 1998), the reduction of T-cell responses observed suggested that insulin B peptide-specific $CD4^+$ Th1 cells were down-regulated by the nasal administration of CTB-insulin B peptide hybrid proteins. It was reported that oral CTB conjugated chemically to insulin suppressed the development of diabetes on NOD mice by regulatory $CD4^+$ Th2 type T cells via the inhibition of pro-inflammatory Th-1 type response (Ploix et al., 1999; Aspord and Thivolet, 2002). The ability of $CD4^+$ T cells to suppress the adoptive transfer of diabetes was explained as bystander suppression mediated by Th2 anti-inflammatory cytokines (Ploix et al., 1999; Aspord and Thivolet, 2002). The insulin peptide-specific $CD4^+$ Th1 cells on present study may be downregulated by the regulatory $CD4^+$ Th2 type T cells.

It was reported that transgenic potatoes that synthesize human insulin and CTB-insulin fusion protein at levels up to 0.05% and 0.1% of total soluble protein, respectively (Arakawa et al., 1998). Feeding transgenic potato tissues producing CTB-insulin fusion protein but not insulin provided a significant reduction in insulitis or diabetic symptoms. It was considered that the plant-synthesized CTB-insulin fusion protein is effective at doses 100-fold less than generally reported for unconjugated autoantigens (Arakawa et al., 1998). In present study, we produced a recombinant CTB fusion protein linked with insulin B chain peptide 9–23 (C19S) at levels up to 200 mg/L culture media in *B.choshinensis* secretion-expression system. Nasal administration of the recombinant *B.choshinensis*-derived hybrid protein of CTB and insulin B peptide suppressed the development of autoimmune diabetes on NOD mice. The recombinant hybrid of CTB and insulin B peptide increased its tolerogenic potential for nasal administration by up 100-fold on molar base of autoantigen peptide. Taken together with the present study, these findings provided new evidence that a hybrid molecule developed from molecule recombinant CTB and insulin or insulin T-cell epitope may be safe and useful in mucosal immune therapy allowing for the induction of mucosally induced

tolerance to auto-reactive T cells in patients with IDDM.

In summary, we produced a uniformed form of hybrid molecule of CTB and insulin B chain peptide at highly expression level by use of *B.choshinensis* recombinant protein expression system. Further, nasal immunization with low doses of the hybrid protein effectively reduced the development of on-going autoimmune diabetes. These results suggest that nasal tolerance produced by administration of the recombinant *B.choshinensis*-hybrid protein of CTB and autoantigen T-cell-epitope could be useful in the control of T-cell-mediated autoimmune diseases such as autoimmune diabetes.

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Mucosa-Associated Lymphoid Tissues in the Aerodigestive Tract: Their Shared and Divergent Traits and Their Importance to the Orchestration of the Mucosal Immune System

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Abstract: As inductive tissues for the initiation of antigen-specific T and B cell responses, the various mucosa-associated lymphoid tissues (MALT) of the aerodigestive tract, which include gut-associated lymphoid tissue (GALT), nasopharynx-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT), share many histological and immunological characteristics. However, recent advances in our molecular and cellular understanding of immunological development have revealed that the various types of MALT also exhibit different molecular and cellular interactions for their organogenesis. In this review, we delineate the distinctive features of GALT, NALT and BALT and seek to show the role played by those features in the regulation of mucosal tissue organogenesis, the mucosal immune system, and mucosal homeostasis, all in an attempt to provide insights which might lead to a prospective mucosal vaccine.

INTRODUCTION

Mucosal surfaces of the gastrointestinal and respiratory tracts represent the principal entry site of a multitude of viral and microbial pathogens. To protect mucosal sites from these invasions, the aerodigestive tract is equipped with multiple physical, biochemical and immunological barriers. The immunological barrier consists of both innate and acquired immunity, with the latter characterized by the initiation of antigen-specific immune response in mucosa-associated lymphoid tissues (MALTs) including the gut-associated lymphoid tissue (GALT), the nasopharynx-associated lymphoid tissue (NALT), and the bronchus-associated lymphoid tissue (BALT) [1, 2]. In particular, Peyer's patches (PPs) and NALT are thought to be representative MALT in the gastrointestinal and respiratory tract, respectively. Additionally, isolated lymphoid follicles (ILFs), which are located throughout the intestine, have been identified and characterized as an additional organized lymphoid tissue in the digestive tract [3]. These tissues contain an interfollicular area that is abundant in T lymphocytes and in high endothelial venules (HEVs), as well as a germinal center (GC), characterized by a dense network of follicular dendritic cells, providing a source of antigen-primed IgA-committed B cells. They also are overlaid by a follicle-associated epithelium (FAE), which contains antigen-sampling M (microfold) cells, allowing selective transport of antigen from the lumen to underlying antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages. Based on these anatomical and histological characteristics of MALT, it has been generally considered that MALTs act as inductive tissues for the generation and priming of antigen-specific T and B cell responses, and that they communicate with effector tissues (e.g., intestinal lamina propria and nasal passages) via an immunological intranet known as the common mucosal immune system (CMIS) [4].

However alike in terms of anatomy and histology, the MALTs of the respiratory and intestinal immune system differ in their organogenesis. Recent studies revealed several key cytokines regulating MALT organogenesis including tumor necrosis factor (TNF) family, lymphotoxin (LT) $\alpha 1\beta 2$/LTβR, and IL-7 receptor α (IL-7Rα) [5]. Additionally, transcriptional regulators, retinoic acid-related orphan receptor γ (RORγ) and inhibitor of DNA binding/differentiation 2 (ID2), shown to play a role in the MALT organogenesis [6, 7]. These discoveries enabled us to paint a detailed picture of the early steps of the development of MALT, especially for PPs and NALT [1, 5]. Surprisingly, though PPs and NALT showed many structural and immunological similarities, they proved to have distinguishable molecular pathways for organogenesis. For example, NALT formation was detected in mice lacking PPs [8]. Additionally, experiments using MALT-deficient mice have revealed the presence of a MALT-independent (an presumably, a CMIS-independent) pathway for the initiation of antigen-specific immune responses [9].

Prompted by these recent findings, we set out to explore whether this new understanding of MALT biology requires us to revisit our assumptions about

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the roles of the various MALTs in the induction and regulation of mucosal homeostasis, including both active and quiescent immune responses. This review attempts to delineate both the traits which link and those which distinguish GALT, NALT and BALT and to explore how the differences may help determine their specific anatomical, developmental and immunological contributions to the mucosal immune system. We will show how this recent progress in our understanding of the structure and function of the various MALTs might facilitate the development of a prospective mucosal vaccine.

THE STRUCTURE AND COMPOSITION OF MALT

The secondary lymphoid tissues are located at immunologically and anatomically important sites where foreign antigens can be presented efficiently to the immune system for the generation of antigen-specific cell-mediated and/or humoral immunity. Systemic immune responses are governed by the anatomical compartment which includes spleen and lymph node (LN), and mucosal immune responses by the compartment including MALT (NALT, BALT, and GALT). These lymphoid compartments share striking similarities in cellular organization, such as discrete T and B cell areas, yet each also has unique characteristics.

Located in the small intestinal anti-mesentery site which extends along the entirety of the small intestine, PPs act as one of the representatives of GALT. They are large enough to be observed upon gross examination, and usually number 8-10 in murine small intestine. PPs are unique in that they contain efferent lymphatics but no afferent lymphatics, reducing the possibility that an antigen will be encountered via the afferent lymphatics. Instead, they take up antigen via a specialized dome region covered with FAE containing M cells [10, 11]. M cells, which are scattered among absorptive epithelial cells (ECs), are distinguished by features such as poorly developed brush borders, reduced degradation activity, and a thin overlying glycocalyx. Additionally, M cells are characterized by a pocket structure (M cell pocket) containing DCs, macrophages, and lymphocytes [12]. Thus, M cells act as a gateway to the outside environment, delivering antigenic substrate to the underlying immune-competent cells for the subsequent induction of antigen-specific immune responses. DCs are present not only in the M cell pocket, but also in the region beneath of FAE (known as the subepithelial dome, SED) [13]. Recent studies demonstrated that $CD11b^+$ DCs were recruited by CCL20 and CCL9 highly expressed by FAE [14, 15]. PPs also contain all of the cellular and microarchitectural elements necessary for the generation of IgA-committed B cells and antigen-specific T cells. At the center of the dome region of PPs lie the B cell follicles, which surround the germinal center (GC) lying at the base of the dome. Unlike the other systemic lymphoid tissues that require infection or local immunization for GC formation, GC is always present in PPs. Because germ-free mice present PPs but lack GCs, it has been postulated that the continuous exposure of the PPs to the commensal bacteria and/or viruses of the outer environment cause the constant GC formation in PPs [16]. At the outside of the dome region, there are interfollicular regions populated by many T cells, and mature DCs but by only a few B cells. These interfollicular regions contain HEV, a main entry site for cells into PPs. The HEV express mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), a ligand for the $\alpha4\beta7$ integrin, which determines a selective migration of gut-tropism lymphocytes [17]. The T cell-rich interfollicular regions overlap with the B cell follicles in some areas, providing an important place for initial T-B interaction [18].

Recently, an additional lymphoid structure resembling PP in composition and architecture has been identified as 100-200 clusters on the antimesenteric wall of the murine small intestine, which is now known as ILF [3]. However, ILF is much smaller than PP (e.g., ILF is about 200 μm and PP is about 3 mm in diameter). They possess an overlying M cells-containing FAE and a large B cell area including GCs. Although lacking a T cell-rich interfollicular region, they do still contain some T cells. These data suggest that ILFs are alternative inductive sites for mucosal immune responses [3].

Another recently discovered feature of the murine intestinal compartment is the Cryptopatch (CP) [19]. CPs are tiny but numerous lymphoid tissues (1500-2000 CPs/intestine) in the cryptal region of the small and large intestine. $c\text{-}kit^+IL\text{-}7R^+Thy1^+Lin$ (CD3, B220, Mac-1, Gr-1 and TER-119)$^-$ lympho-hematopoietic progenitors have been shown to accumulate in CP. CP has also been considered as a site where extrathymic differentiation of T cells occurs for the intraepithelial lymphocytes (IELs). To this end, it has been shown that T cell receptor (TCR) $\alpha\beta$ and TCR$\gamma\delta$ IELs were developed by in vivo transfer of CP progenitor into severe combined immunodeficient (SCID) mice [20].

The respiratory tract is another site characterized by mucosa-associated lymphoid structures. In rodents, NALT is found on both sides of the nasopharyngeal duct dorsal to the cartilaginous soft palate and is considered analogous to the Waldeyer's ring in humans [21]. Furthermore, a NALT-like structure of lymphocyte aggregation with follicle formation was identified in human nasal mucosa, especially that of the middle concha in children less than two years old [22]. Like PPs in the intestine, NALT has been considered as an inductive tissue for the nose and exhibits FAE where antigen-sampling M cells are present [23]. NALT is also composed of follicles and interfollicular regions which are B cell- and T cell-enriched sites, respectively. Characterization of messenger RNA encoding helper

T (Th) 1 and Th2 cytokines in $CD4^+$ T cells isolated from murine NALT revealed a dominant Th0 cytokine profile, indicating that these T cells are capable of becoming Th1 or Th2 cells immediately after antigen exposure through the nasal tract [24, 25]. In the interfollicular regions, NALT-associated HEVs express peripheral node addressin (PNAd), which is regulated by the LTβR- NF-κB-inducing kinase (NIK)-IKKα signaling pathway [26]. APCs including DCs and macrophages are also found in NALT [27]. Thus, NALT contains all of the lymphoid cells necessary for the induction and regulation of mucosal immune responses to nasally exposed antigens.

In 1973, Bienenstock *et al.* described that BALT was classically defined as an aggregated lymphoid structure separated from the bronchial lumen by a specialized lymphoepithelium [28, 29]. Although the presence of murine and human BALT as an aggregated lymphoid follicle remains a subject of debate, BALT has been described in several species including rats, rabbits, and sheep [30, 31]. Similar to the PPs and NALT, BALT does not draw upon afferent lymphatics for antigen retrieval. Instead, it samples antigen directly from the lung lumen through M cells [32], with HEV being the only entry site for lymphocytes into BALT [33]. The migration of the lymphocytes from the blood through HEV is possibly mediated in part by the L-selectin ligand, PNAd [34]. This lymphoid architecture was characterized by the presence of CXCL13 and CCL21 in and around germinal centers, respectively, for IgA antibody production [35]. Another study indicated that lung $CD11c^{bright}$ DCs were the principal APCs to uptake antigens in lung following intranasal immunization, resulting in the direct priming of $CD4^+$ T cells that did not require their migration into draining LNs [36]. These immunological and histological features of BALT in experimental animals suggest that it resembles the other mucosal organized lymphoid structures that induce protection against respiratory infections.

DISTINCT PATHWAY FOR MALT ORGANOGENESIS

The Molecular and Cellular Mechanisms of GALT Organogenesis

Recently, a model describing the development of PP was proposed based on the molecular and cellular characterization of IL-7R, LTβR and chemokine receptor-mediated tissue genesis (Fig. **1**) [37]. It was shown that lymphoid-lineage $IL\text{-}7R^+CD3^-CD4^+CD45^+$ cells are considered to be PP inducers expressing CXC chemokine receptor 5 (CXCR5) and can produce membrane-associated $LT\alpha1\beta2$ heterotrimer, whereas mesenchymal-lineage $VCAM\text{-}1^+$ and intercellular adhesion molecule (ICAM)-1^+ PP organizers express LTβR [38, 39]. The profile of receptor expression suggests the presence of reciprocal signaling cascades between the inducer and organizer cells. Following stimulation signals provided through IL-7R, PP inducers express $LT\alpha1\beta2$ to activate PP organizers through LTβR, and PP organizers produce chemokines such as CXCL13 and CCL19 to stimulate PP inducers through CXCR5 and CCR7 [40]. The reciprocal interaction between inducer and organizer cells through the chemokine and cytokine receptors is essential for the initiation of PP formation, and the loss of any part of the signaling program is sufficient to disrupt secondary lymphoid tissue development, as evidenced by the loss of PP in $LT\beta R^{-/-}$ and $IL\text{-}7R\alpha^{-/-}$ mice [41, 42]. Furthermore, deletion of the gene encoding CXCR5 resulted in a partial reduction in the formation and number of PP [43]. The lack of PP and lymph nodes in *aly/aly* (or alymphoplasia) mice, which have defective NIK function, also fits this paradigm, as recent analyses have established that NIK is essential for the transduction of signals through the TNFR family, including those through LTβR [44, 45]. Thus, *aly/aly* mice lack PP as the NIK mutation inhibits the reciprocal interaction between PP inducers and organizers through $LT\alpha1\beta2$ and LTβR [46]. Further evidence in support of this model comes from studies showing that mice lacking the $CD3^-CD4^+CD45^+IL\text{-}7R^+$ inducer cells due to genetic deletion of the transcription regulators Id2 or RORγ also completely lack the formation of PP and LN [7, 47].

The formation of ILF also requires LT- and LTβR-dependent events [3]. However, unlike PP formation, the LT- and LTβR-dependent events required for ILF formation can occur postnatally and require LT-expressing B lymphocytes [48]. Consistent with this fact, treatment with LTβR-Ig fusion protein during the postnatal period results suppresses ILFs but not PPs [49]. It was also demonstrated that immature ILFs with clusters of $B220^+$ cells were present in the intestine of germ-free mice, but exogenous stimuli including bacterial antigens/mitogens are required for the completion of the lymphoid organization of ILFs including GC formation [48]. Consistent data was obtained in the mice lacking activation-induced cystidine deaminase (AID) that is an important molecule for class switch recombination (CSR) as mentioned below [50]. In the AID-deficient mice, the ILFs developed hyperplasia. A recent study demonstrated that the lack of hypermutated IgA production into the intestinal lumen resulted in the expansion of segmented filamentous bacteria in the small intestine of AID-deficient mice. Because antibiotic treatment of AID-deficient mice abolished the hyperplasia, the authors of that study proposed that the anaerobic expansion induced ILF hyperplasia [50, 51]. Taken together, these findings suggest that postnatal and physiological inflammatory signals are essential for the formulation of ILFs.

Membrane-bound $LT\alpha1\beta2$ interactions with LTβR are also essential for CP development. Thus, $LT\alpha^{-/-}$ or $LT\beta R^{-/-}$ mice lack CP [52]. Like ILFs, CP

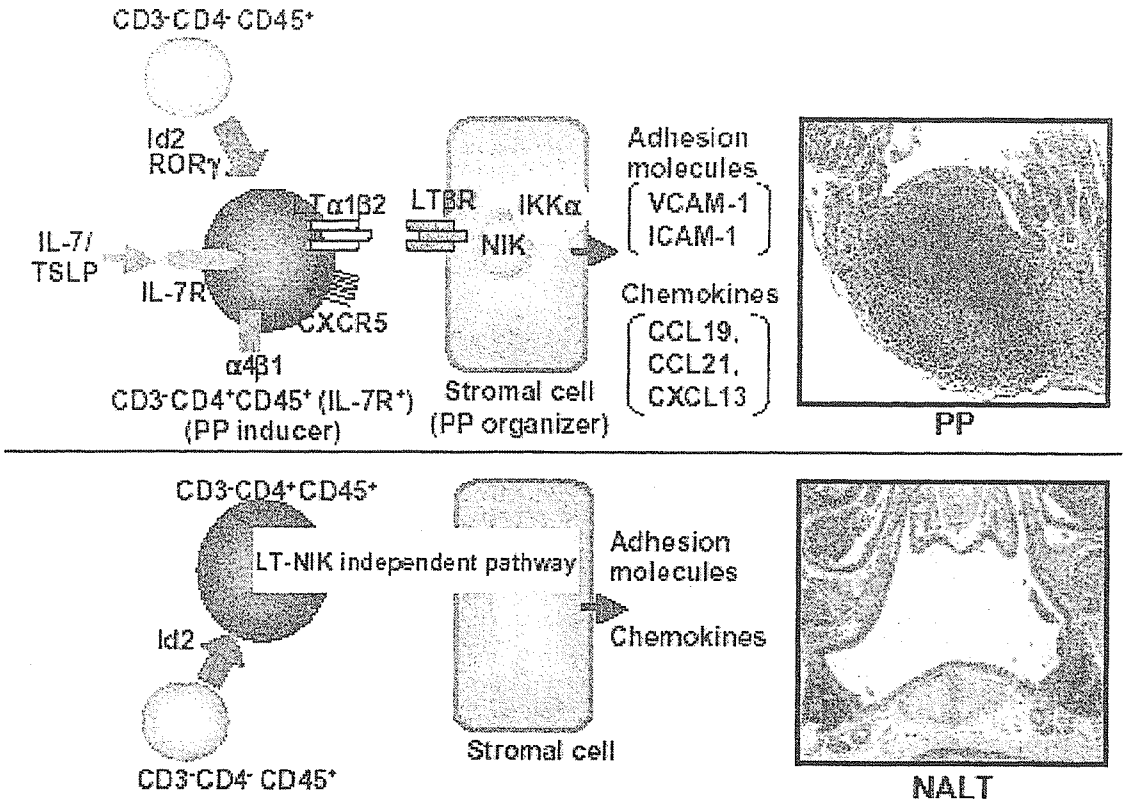

Figure 1. Comparison of the organogenesis programs of NALT and PPs. $CD3^-CD4^+CD45^+$ cells are considered to be the common inducers of secondary lymphoid tissue. Id2 is indispensable for the induction and differentiation of these inducer cells from their fetal liver precursors ($CD3^-CD4^-CD45^+$). In the case of PPs, IL-7 and/or TSLP and IL-7R interaction induces the expression of the LTα1β2 heterotrimer on these $CD3^-CD4^+CD45^+$ PP inducer cells. LTα1β2 heterotrimer binds to LTβR on stromal cells as a PP organizer and induces signal transduction through NIK-IKKα for the expression of necessary adhesion molecules and/or chemokines. CXCL13 produced by a PP organizer activates the form of α4β1 expressed on the CXCR5-positive PP inducer. The activated form of α4β1 contributes to the interaction of the PP inducer and the PP organizer. Thus, IL-7R- and LTβR- mediated signals are essential for the tissue genesis of PPs. The development of $CD3^-CD4^+CD45^+$ cells in NALT also requires Id2; however, the initiation of NALT organogenesis is independent of IL-7R, LTα1β2/LTβR and NIK signaling.

development seems to be a postnatal event. Thus, CPs were fist detected at 14-17 days after birth [19]. However, CPs are present in the small intestine of *aly/aly* mice that do not contain PPs and ILFs [52]. These results indicate that an alternative LTβR and NF-κB activation pathway may play a crucial role in the development of CP.

Distinct Features of NALT Organogenesis Compared with PP

In normal mice, NALT is a bell-shaped tissue characterized by the accumulation of a variety of lymphoid cells that are capable of initiating both humoral and cellular immunity. NALT formation has not been observed during embryogenesis or in newborn nasal tissue. $PNAd^+$ HEV is first detected in bilateral nasal tissue one week after birth, and the complete bell-shaped NALT formation with lymphoid cells eight weeks after birth [8]. These findings indicate the presence of a distinct tissue genesis program in the intestinal and respiratory immune systems, where a pre-natal initiation of lymphoid organogenesis occurs for PP and NALT, respectively. An intriguing possibility is that the NALT genesis program is triggered after birth by stimulation signals provided by environmental antigens and mitogens. However, we have observed the formation of NALT in adult mice born and raised under germ-free conditions (unpublished results). A similar situation was also seen in germ-free rats [53]. Thus, one possibility could be that the initiation of NALT genesis is programmed to be activated after birth and the subsequent maturation process is controlled by environmental antigens.

PP formation requires a cytokine-signaling cascade involving IL-7R and LTβR (Fig. **1**). However, organized nasal lymphoid tissue was detected in all mouse strains lacking PP or both PP and LN due to a deficiency in the LTβR-mediated inflammatory cytokine cascade, including $LT\alpha^{-/-}$, $LT\beta^{-/-}$, and *aly/aly* mice, and mice treated *in utero* with LTβR-Ig fusion protein [8, 54]. These findings further support the idea that NALT tissue genesis does not follow the "programmed inflammation" model typical of PP. We

next examined NALT development in IL-7R$^{-/-}$ mice, as both the IL-7R-mediated signaling pathway as well as the LTβR cascade have been shown to be requisite for PP development. NALT, but not PP, did develop in IL-7R$^{-/-}$ mice [8]. Taken together, these findings directly demonstrate that NALT formation is independent of IL-7R- and LTβR-mediated tissue genesis.

A unique subset of mononuclear cells characterized as CD3$^-$CD4$^+$CD45$^+$ have been shown to act as inducer cells for the organogenesis of secondary lymphoid tissues including PP in the intestinal wall (Fig. **1**) [40]. Furthermore, Id2 has been identified as one of the genes responsible for the induction of these CD3$^-$CD4$^+$CD45$^+$ inducer cells [7]. Not surprisingly then, deletion of the Id2 gene completely impaired the genesis of all secondary lymphoid tissues including both NALT and PP [7, 8]. In normal and LT?$^{-/-}$ mice, these CD3$^-$CD4$^+$CD45$^+$ cells accumulate to form the NALT anlagen. Adoptive transfer of fetal liver cells or CD3$^-$CD4$^+$CD45$^+$ cells into Id2$^{-/-}$ newborn mice could generate a NALT-like structure in these mice (Fig. **1**). [8]. The transcriptional regulator ROR? has also been shown to be required for the development of CD3$^-$CD4$^+$CD45$^+$ inducer cells [47]. When the gene encoding RORγ was deleted, PP and LN organogenesis was suppressed [47]. However, NALT development has been reported in RORγ-deficient mice [54]. These findings suggest that although NALT and PP share the same phenotype of inducer cells, CD3$^-$CD4$^+$CD45$^+$, those inducer cells can be categorized into those dependent on Id2 alone (like NALT) and those dependent on both Id2 and RORγ (like PP)[1].

Development of BALT

As mentioned above, BALT appears in some but not all vertebrate species and the timing of its appearance varies among species as well. Classical BALT appears during the late embryonic development in the rat and the pig, but not in the human and the mouse [30, 31]. However, several lines of evidence suggest that *de novo* organization of lymphoid tissues and GCs are induced in murine and human lung by infection, immunization or inflammation [55, 56]. To distinguish these immunological reaction-induced structures and formation pathway from those of classical BALT, they have been termed inducible BALT (iBALT). A recent study indicated that iBALT structures were formed independently of LTα expression and demonstrated that mice lacking spleen, LNs, and PPs, but having iBALT, exhibited substantial T cell- and B cell-mediated immune responses to clear influenza infection, which seems to be mediated by iBALT [35]. However, it is also possible that NALT may contribute to the original phase of the induction pathway of iBALT, since the inhaled antigens first make contact with NALT prior to their deposition on BALT. Also, since antigen-activated lymphocytes are first observed at the lymph nodes (e.g., NALT and CLN) that drain the respiratory tract and are subsequently found in the lung, it is possible that iBALT is just an accumulation site for the effector cells that are initially primed in conventional lymphoid organs (e.g., NALT) [57]. Thus, both the organogenesis of iBALT and its contribution to the priming and expansion of the T and B cell responses remain obscure, and further study is required to clarify this important issue.

INITIATION OF ANTIGEN-SPECIFIC IMMUNE RESPONSES IN MALT FOR THE COMMON MUCOSAL IMMUNE SYSTEM (CMIS) PATHWAY

The M cell: a Unique Antigen-Sampling Cell

Although the organogenesis pathways for PP, ILF and NALT are quite distinct above, they all act as inductive tissues for the initiation of antigen-specific immune responses via the CMIS-dependent pathway (Fig. **2**). First, epithelia covering the inductive tissues develop an FAE containing scattered professional antigen-sampling M cells to achieve selective transport of foreign antigen from the lumen into the inductive tissues [3, 11, 23]. The M cells can be distinguished from the surrounding ECs by their unique histological and biological features, including a lack of brush borders, a limited mucus production, and a unique basolateral invagination that creates a pocket containing immunocompetent cells such as DCs, macrophages, T cells and B cells [12]. M cells also show lower degradation and higher transcytosis activity than do the neighboring ECs, and they express receptors for some microorganisms [10]. For example, *Yersinia* adheres to M cells via the invasin and the β1 integrin expressed on the *Yersinia* and M cells, respectively, allowing the selective invasion through M cells [58]. Thus, when invasion was mutated, Yersinia could no longer adhere to and invade M cells [58]. Similarly, when reovirus is ingested orally, intestinal proteases modify its outer capsid membranes (sigma 3 and μ1c), thereby inducing the expression of σ1 protein containing a sialic acid-specific, lectin-like domain which is specific for glycoconjugates containing α2-3-linked sialic acid expressed on the apical side of M cells [59]. These characteristics of M cells allow them to selectively transport antigens and microorganisms from the lumen to the APCs in the M cell pocket..

Priming by Mocosal APCs of Naïve T Cells and B Cells in Inductive Tissues

Once APCs in the M cell pocket take antigen up from the intestinal lumen through the mediation of the M cell, they process the antigen and migrate into the interfollicular areas of the PPs, where they present antigenic epitopes with major histocompatibility complex (MHC) molecules (Fig. **2**) [14, 60]. Although it has been already reported that NALT DCs present antigen after intranasal

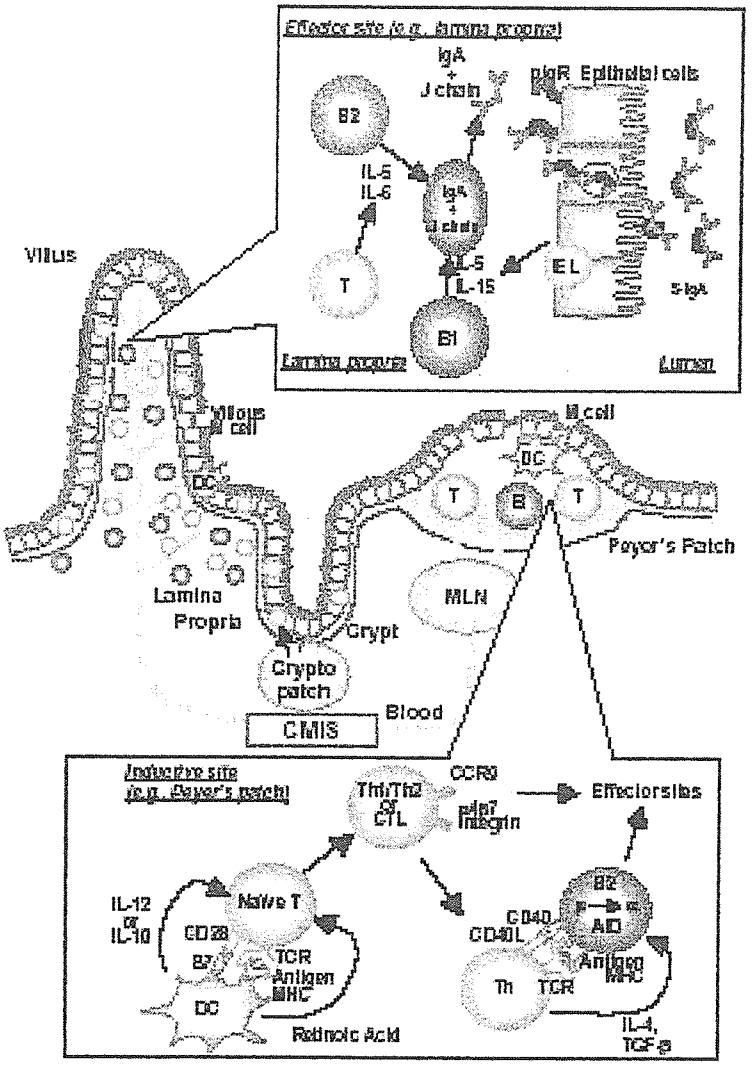

Figure 2. Multi-layered barriers provided by innate and acquired immunity in the intestine. Following antigen transport through M cells, DCs obtain the antigen and present it to naïve T cells, causing them to differentiate into Th or CTL cells in the presence of DC-produced IL-10 or IL-12, respectively. Retinoic acid derived from DCs determines the T cell tropism to migrate to mucosal effector sites by the up-regulation of CCR9 and α4β7 integrin on T cells. Th cells interact with B2 cells to induce CSR, which induces IgA-committed B cells. This step is mediated by the CD40/CD40 ligand interaction, and by IL-4 and TGF-β. These antigen-specific T and B cells migrate preferentially into mucosal effector sites (e.g., lamina propria) through HEV. Mucosal T cell-derived IL-5 and IL-6 promote differentiation of IgA-committed B2 cells into IgA plasma cells, resulting in the production of polymeric IgA (IgA+J chain). The polymeric IgA is associated with the expression of pIgR on the basal side of epithelial cells followed by the production of secretory IgA (S-IgA) into the lumen. At the mucosal effector sites, B1 cells are also present and are activated by EC-derived IL-15 to differentiate into plasma cells for the secretion of IgA against common bacterial products like phosphatidylcholine. Like the IgA originating from B2 cells, the B1 cell-derived IgA is also transported into the lumen as a form of S-IgA. Some populations of IELs located between the ECs recognize non-classical MHC molecules on ECs that are induced by some bacterial and/or viral infections. Thus, B1 cell-derived IgA and IEL have an ability to react to the antigen without any fine specificity for microorganisms, and thus may play a crucial role in the innate aspect of mucosal immunity.

immunization [27], detailed pathway for the antigen uptake and their migration in NALT is still unclear. Also unclear is the mechanism by which the newly identified and characterized ILF in the intestinal tract prime Th cells and B cells via DCs. The antigen presentation by APCs stimulates naïve T cells which predominate in the inductive tissues, resulting in the differentiation of naïve T cells into Th1, Th2 or cytotoxic T lymphocytes (CTLs) [24, 60, 61] (Fig. **2**). To this end, three distinct populations of DCs were reported in PPs [14]. $CD11b^+$ DCs are capable of producing IL-10 by the stimulation of the CD40 ligand, whereas $CD8^+$ DCs or $CD11b^-CD8^-$ (double negative) DCs produce IL-12 by microbial stimulation, resulting in the induction of Th1 and Th2, respectively [60].

The cross talking among DCs, T cells, and B cells at inductive tissues promotes the IgA-commitment of B cells, which undergo μ to α isotype CSR [62, 63] (Figs. **2** and **3**). This μ to α isotype CSR is likely dependent on antigen stimulation at GCs, where naïve B cells interact with local $CD4^+$ T cells and with follicular antigen-trapping DCs. Key interactions required for this switch include ligation of CD40 on B cells with the CD40 ligand on Th cells [64], the presence of transforming growth factor (TGF)-β [65, 66], and the interaction between the inducible co-stimulator (ICOS) expressed on activated Th cells and its ligand ICOS-L constitutively expressed by B cells [67]. Stimulated by these interactions, multiple transcription factors are activated and promote the process of CSR (Fig. **3**). For example, transcriptional activation of the $C_H\alpha$ gene requires the TGF-β-mediated activation of Smad3/4 and the *de novo* synthesis of core-binding factor (CBF) α3 that binds to the Smad-binding elements (SBEs) and CBF sites on the intronic $C_H\alpha$ promoter region [68, 69]. A dramatic breakthrough in our understanding of CSR came with the discovery of AID [70, 71]. AID, which exhibits a single-stranded DNA deaminase activity, is expressed in activated B cells, particularly in the GC [72]. It associates with the CSR target chromatin in a germline transcription-coupled manner [73]. As might be expected given the ability of PPs, ILFs, and NALT to induce the generation of IgA-committed B cells, AID expression and Iα-Cμ circle transcripts and their reaction products were always detected at these inductive tissues [63].

In contrast to the dominant class switch to IgA in PPs, B cell differentiation in NALT was shown to lead to the production of both IgA and IgG [74]. It had

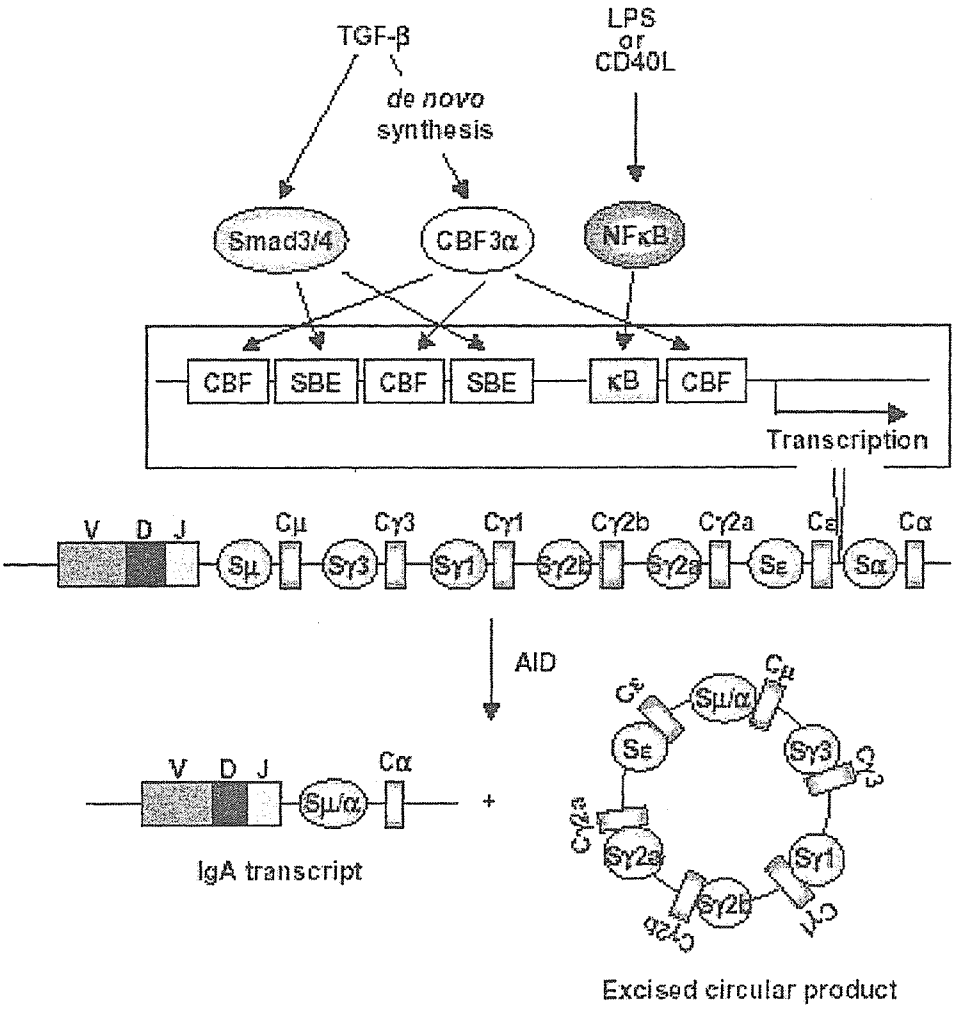

Figure 3. Molecular events for class switching to IgA. A secondary isotype of Ig is produced by CSR, a process for exchange of the constant (C) region of the heavy chain. This pathway requires AID activity for the DNA break in the switch (S) region, which is mediated by DNA deamination. Following DNA repair, it generates rearranged DNA encoding a different isotype of Ig and the deletion of the intervening sequence as a circular DNA. TGF-β-induced Smad3/4 binds to the SBE region on the Cα promoter and the TGF-β-mediated signaling also induces *de novo* synthesis of CBFα3 that is in turn capable of binding to the CBF region on the Cα promoter. These bindings facilitate the transcription of the Cα region, presumably in the presence of the NFκB-mediated κB activation, determining the class switch to IgA.

been previously established that the development of IgA-committed cells in the presence of TGF-β was characterized by sequential CSR from Cμ to Cα via Cγ, a pathway mediated by CD40 engagement [64, 75]. It is also interesting to note that human tonsils have been shown to contain a high frequency of IgG B cells in addition to IgA [76, 77]. These findings may explain the equal commitment of B cells to IgG and to IgA in NALT, but further analysis will be required to reveal the molecular mechanism involved in the generation of mucosal B cells with those two different isotypes.

Egress from Inductive Sites and Migration to Effector Sites

After egressing from PP and NALT, the IgA-committed B cells migrate to mesenteric lymph nodes (MLN) and cervical lymph nodes (CLN), respectively. Accumulating evidence suggests that the egress of B cells and T cells from PP is restricted by sphingosine-1-phosphate receptor type 1 (S1P1) (Fig. **4**) [78, 79]. Thus, treatment of mice with FTY720, an agonist of S1P1, induced dramatic reduction in S1P1 expression, inhibiting lymphocyte egress from PPs and resulting in the accumulation of B and T cells in PPs.

The IgA-committed B cells migrate preferentially into the mucosal effector tissues (e.g., the gut lamina propria and the nasal passage) through the thoracic duct and blood circulation operated by the CMIS (Figs. **2** and **4**). Several lines of evidence suggest that the trafficking of IgA-committed B cells to the gut lamina propria is facilitated by changes in the expression of adhesion molecules and chemokine receptors. Changes in adhesive molecules include α4β7 integrin [80, 81], which is used to MAdCAM-1-mediated traffic into the intestinal effector site [17]. CCR9, a later chemokine receptor is selectively expressed on IgA-, but not IgM- or IgG-, committed B cells and their ligand, CCL25 (also known as thymus-expressed chemokine TECK) is produced predominantly by the intestinal epithelium [82]. These combinations of homing receptors and chemokine receptors conclude the selective homing

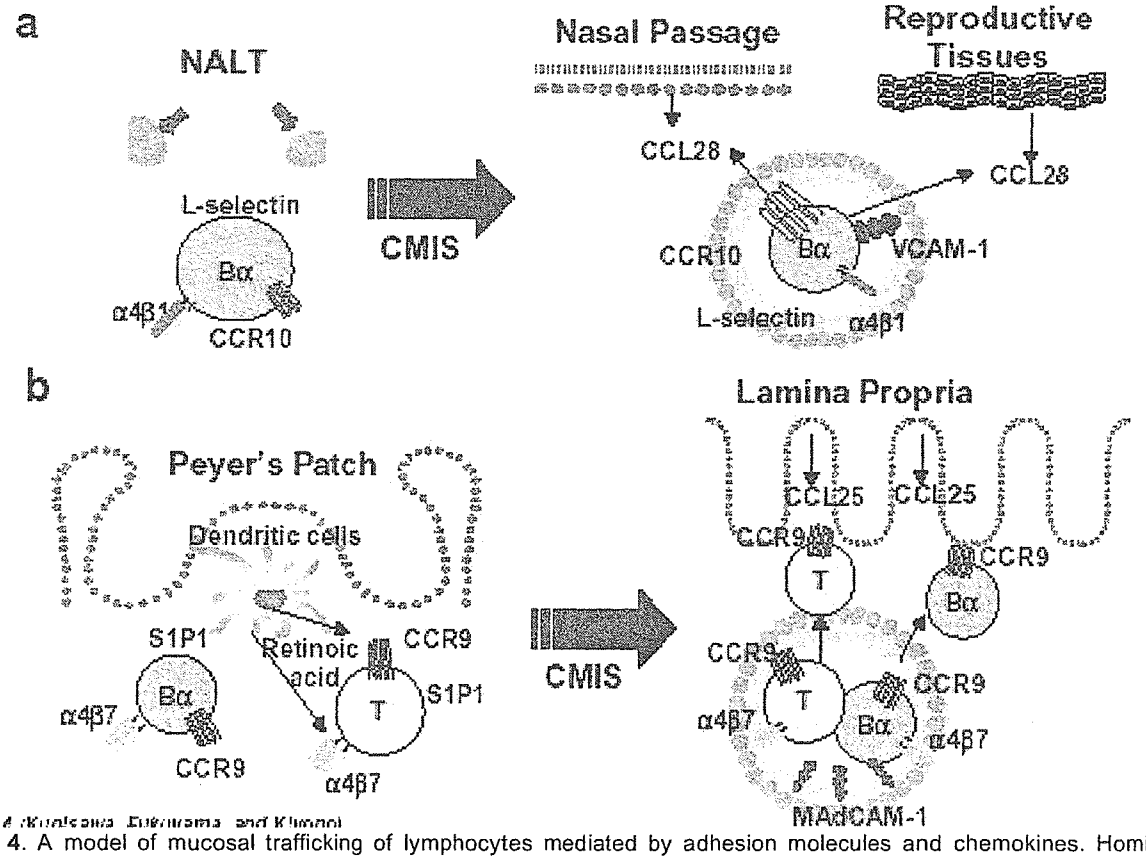

Figure 4. A model of mucosal trafficking of lymphocytes mediated by adhesion molecules and chemokines. Homing of lymphocytes into mucosal effector sites requires and is operated by several adhesion molecules and chemokines. (a) IgA-committed B (Bα) cells developed in NALT express α4β1 integrin and CCR10. Through CMIS, these IgA-committed B cells migrate into nasal passages and reproductive tissues (e.g., vagina) by the interaction of α4β1 integrin/VCAM-1 and CCR10/CCL28. (b) In the case of PP, S1P1 is essential for the egress of T cells and IgA-committed B cells. Retinoic acid produced by DCs induces the expression of α4β7 and CCR9 on antigen-primed T cells. MAdCAM-1$^+$ HEV in intestinal lamina propria substantially contribute to the homing of α4β7$^+$ IgA-committed B cells and T cells from PP. Furthermore, tissue-specific production of CCL25 by intestinal epithelial cells involves the gut-homing specificity of CCR9$^+$ IgA-committed B cells and T cells to the lamina propria region of the intestinal tract.