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A rice-based edible vaccine expressing multiple T cell epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses

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Peptide immunotherapy using multiple predominant allergen-specific T cell epitopes is a safe and promising strategy for the control of type I allergy. In this study, we developed transgenic rice plants expressing mouse dominant T cell epitope peptides of Cry j I and Cry j II allergens of Japanese cedar pollen as a fusion protein with the soybean seed storage protein glycinin. Under the control of the rice seed storage protein glutelin *GluB-1* promoter, the fusion protein was specifically expressed and accumulated in seeds at a level of 0.5% of the total seed protein. Oral feeding to mice of transgenic rice seeds expressing the T cell epitope peptides of Cry j I and Cry j II before systemic challenge with total protein of cedar pollen inhibited the development of allergen-specific serum IgE and IgG antibody and CD4$^+$ T cell proliferative responses. The levels of allergen-specific CD4$^+$ T cell-derived allergy-associated T helper 2 cytokine production of IL-4, IL-5, and IL-13 and histamine release in serum were significantly decreased. Moreover, the development of pollen-induced clinical symptoms was inhibited in our experimental sneezing mouse model. These results indicate the potential of transgenic rice seeds in production and mucosal delivery of allergen-specific T cell epitope peptides for the induction of oral tolerance to pollen allergens.

Japanese cedar pollinosis | peptide immunotherapy | seed-specific expression

Immunotherapy using allergen-specific T cell epitope peptides has been shown to be a safe and effective treatment for the control of IgE-mediated allergic diseases (1–3). Immunodominant epitopes derived from several allergens have been shown to possess therapeutic effects in both animal models and human clinical trials (4–8). Japanese cedar (*Cryptomeria japonica*) pollen is a major cause of pollinosis that elicits allergic disorders such as rhinitis and conjunctivitis in Japan (9). Two major allergens, designated Cry j I and Cry j II, were isolated from the pollen (9–13), and multiple domains of T cell epitope for humans and mice were identified from them (14–16). It has been reported that oral feeding to mice of a chemically synthesized major T cell epitope peptide of Cry j II reduces levels of Cry j II-specific IgE and IgG antibody responses via a decrease in the production of allergy-associated IL-4 in mice (15). These results open new possibilities for the development of allergen peptide-based immunotherapy for the control of Japanese cedar-induced pollinosis. Thus, oral vaccination with the major T cell epitope peptides derived from Cry j I and/or Cry j II pollen allergens is considered to be a practical and effective method of immunotherapy for the inhibition of pollinosis-associated type I hypersensitivity.

Plants have recently been recognized as a form of bioreactor for the cost-effective production of large-scale recombinant proteins (17–19). Compared to other expression systems such as bacteria and mammalian cell cultures, plants have a much lower risk of contamination by human pathogens, such as animal virus and prions (17–19). Furthermore, the edible tissues of plants further provide the significant benefit of achieving a simple method for mucosal delivery of vaccines and immunogens without the need for complicated purification steps (20–22).

Cereal crop seeds are essentially edible tissues and have the capacity to produce relatively large amounts of recombinant products (23, 24). Recombinant products accumulated in seeds have been shown to be stable for 6 months, even when stored at room temperature (19). Rice, a staple food in Asia, can be considered as an attractive system, compared to other cereals, because of its easy storage and processing, high yield, and low production cost (25). A detailed search for a number of promoters, using β-glucronidase (GUS) reporter gene, provided a choice of suitable promoters for the effective expression of transgenes in rice seeds (26). Another advantage of rice plants is that targeting to protein storage vacuoles (protein bodies) provides a greater space for the accumulation of recombinant proteins (27). A soybean glycinin A1aB1b provided one successful instance of high-level accumulation in the protein storage vacuole II (protein body II), reaching ≈5% of the total seed protein (27). Furthermore, the expression level of glycinin A1aB1b was enhanced in low storage protein mutants of rice (28). Based on the progress of molecular analysis of the expression and accumulation of transgene products, rice can be considered a potential candidate for the development of plant-derived edible vaccines.

In this study, we developed transgenic rice plants accumulating mouse T cell epitope peptides specific for pollen allergens of *Cryptomeria japonica* in seeds. To achieve greater accumulation, the T cell epitope peptides of Cry j I and Cry j II were expressed as a fusion protein with the soybean storage protein glycinin A1aB1b. The fusion protein (A1aB1b-Crp-1 and -2) accumulated at a level of 0.5% of the total seed protein. Oral administration of the transgenic rice seeds to mice before systemic challenge with total cedar pollen protein induced oral tolerance with the inhibition of allergen-induced allergy-associated T helper 2 (Th2) cytokine synthesis of IL-4, IL-5, and IL-13 and their supported allergen-specific IgE responses. Furthermore, it resulted in the inhibition of the pollen-induced clinical symptoms of nasal sneezing. These results demonstrate the efficacy of T cell epitope peptides expressed in transgenic rice seeds for oral delivery and induction of oral tolerance against pollen allergen-specific responses.

Methods

Plasmid Construction and Rice Transformation. Two major T cell epitopes, KQVTIRIGCKTSSS (residues 277–290 of Cry j I) and RAEVSYVHVNGAKF (residues 246–259 of Cry j II) (15, 16),

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Abbreviation: Thn, T helper *n*.

See Commentary on page 17255.

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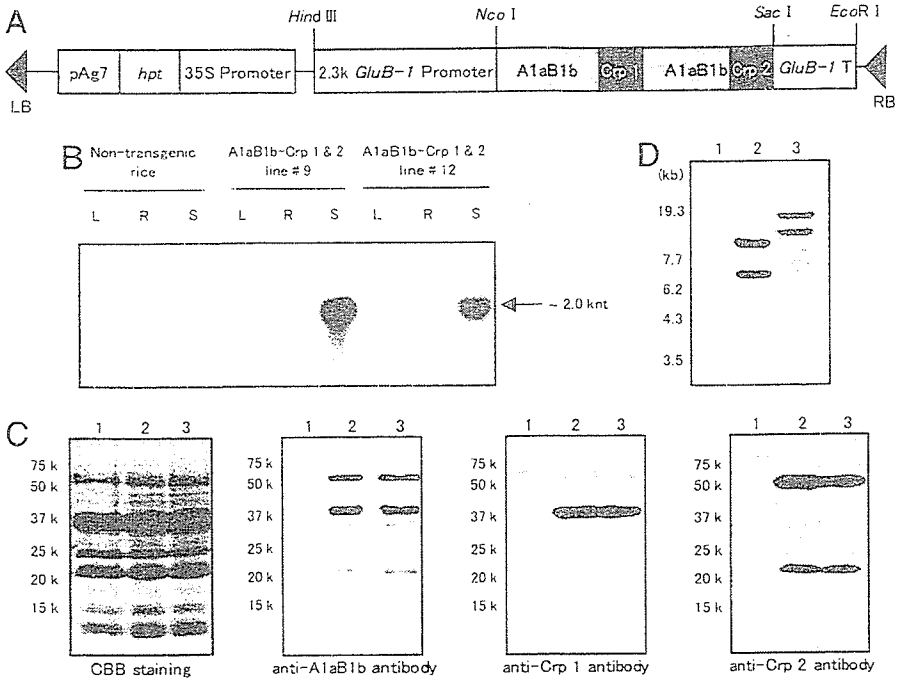

Fig. 1. Expression of A1aB1b-Crp-1 and -2 in transgenic rice. (*A*) Schematic representation of the transformation plasmid. The DNA fragment coding for the A1aB1b-Crp-1 and -2 protein was placed under the control of rice seed major storage protein glutelin 2.3-kb *GluB-1* promoter. The *hpt* gene was used for the selection of transgenic rice plants. *GluB-1*, rice glutelin *GluB-1*; 35S, cauliflower mosaic virus 35S promoter; *hpt*, hygromycin phosphotransferase gene; pAg7, agropine synthase polyadenylation signal sequence; RB, right border; LB, left border. (*B*) Northern blot analysis. Total RNA was isolated from leaves (L), roots (R), or developing seeds (S) of nontransgenic and A1aB1b-Crp-1 and -2 transgenic lines 9 and 12. (*C*) Western blot analysis of total protein extracted from seeds. Lane 1, nontransgenic rice; lane 2, A1aB1b-Crp-1 and -2 transgenic line 9; lane 3, A1aB1b-Crp-1 and -2 transgenic line 12. Anti-glycinin antibody, anti-Crp-1 antibody, or anti-Crp-2 antibody was used for the detection of A1aB1b-Crp-1 and -2 protein. (*D*) Southern blot analysis. Genomic DNA isolated from young leaves of rice plants was digested with SacI and fractionated by electrophoresis on 0.8% agarose gel. Lane 1, nontransgenic rice; lane 2, A1aB1b-Crp-1 and -2 transgenic line #9; lane 3, A1aB1b-Crp 1 and -2 transgenic line 12.

named Crp-1 and -2, respectively, were inserted into variable regions in acidic and basic subunits of glycinin A1aB1b (29, 30). Fifteen amino acid residues (residues 293–307 of A1aB1b) in the acidic subunit and eight amino acid residues (residues 488–495 of A1aB1b) in the basic subunit were substituted by the Crp-1 and -2 T cell epitopes, respectively, resulting in the recombinant protein A1aB1b-Crp-1 and -2. The construction of the A1aB1b-Crp-1 and -2 gene sequence was carried out by two stages of PCR amplification. A DNA sequence coding for the acidic subunit (residues 1–292 of A1aB1b) was amplified by PCR from the pUGluBGly plasmid (27) with a set of oligonucleotides −103 and Crp1R, which added a DNA sequence coding for the Crp-1 peptide at the 3′ end of the acidic subunit of A1aB1b sequence. The other sequence coding for the basic subunit (residues 308–487 of A1aB1b) was PCR-amplified by using the primer set Crp1F and M13-RV, which provided DNA sequences coding for the Crp-1 and -2 peptides at the 5′ and 3′ end of the basic subunit of A1aB1b sequence, respectively. These two DNA fragments were then annealed and amplified by overlap PCR with −103 and M13-RV primers to generate the complete DNA fragment coding for the A1aB1b-Crp-1 and -2 protein. This product was placed under the control of the 2.3-kb *GluB-1* promoter, and the plant expression cassette was then inserted into a binary vector pGPTV-35S-HPT (26). The resultant expression plasmid (Fig. 1*A*) was introduced into the rice genome (*Oryza sativa* L. cv Kitaake) by *Agrobacterium tumefaciens*-mediated transformation as described (26).

Southern and Northern Blot Analysis. Genomic DNA was prepared from young leaves by using the cetyltrimethylammonium bromide (CTAB) extraction method (28). Total RNA was extracted by the phenol/chloroform extraction method (28) from frozen rice seeds, leaves, or roots. Southern and Northern blot analyses were carried out by using standard methods (28). Hybridizations were performed at 65°C by using ^{32}P-labeled full-length A1aB1b-Crp-1 and -2 probes.

Detection of A1aB1b-Crp-1 and -2 Protein. Rice seeds were ground to a fine powder by using a Multibeads shocker (Yasui Kikai, Osaka, Japan), and total seed protein was extracted with an extraction buffer containing 4% (wt/vol) SDS, 8 M urea, 5% (wt/vol) 2-mercaptoethanol, 50 mM Tris·HCl (pH 6.8), and 20% (wt/vol) glycerol as described (28). Total seed protein was separated by using SDS/12% or 15% PAGE, and then transferred to Hybond-P poly(vinylidene difluoride) membranes (Amersham Pharmacia) for Western blot analysis. To confirm the accumulation of Crp-1 and -2 T cell epitope peptides in transgenic rice seeds, anti-Crp-1 peptide and anti-Crp-2 peptide antibodies were raised in rabbit (Qiagen, Tokyo). A rabbit anti-glycinin A1aB1b antibody had been prepared previously (27). The membranes were probed with one of the primary antibodies, and then incubated with a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Promega) for visualizing signals. Accumulation levels of A1aB1b-Crp-1 and -2 protein were determined by the quantitative dot immunoblotting with anti-glycinin A1aB1b antibodies as described (27).

Mouse Feeding Experiments. A first group of eight BALB/c male mice at 6 weeks of age (CLEA Japan, Tokyo) was orally fed with 200 mg of fine powder of rice seeds containing 70 μg of A1aB1b-Crp-1 and -2 protein suspended in 1.0 ml of PBS once a day over a period of 4 weeks. A second group of mice was fed with equal amounts of seed powder from nontransgenic rice plants. For a third group of mice, PBS was administered as a control experiment. All mice were then i.p. challenged twice at weeks 4 and 5 with 0.1 mg of total protein extracts of Japanese cedar pollen (Cosmo Bio, Tokyo) adsorbed on 5 mg of aluminum hydroxide (alum) (Cosmo Bio) in 500 μl of PBS. At the first challenge at week 4, recombinant mouse IL-4 (R & D Systems) was mixed with the allergen solution at 0.1 μg per mouse to maximize the induction of allergen-specific IgE responses. Our preliminary study demonstrated that the co-administration of IL-4 resulted in the acceleration of allergen-specific IgE responses compared with the case when IL-4 was not coadministered.

ELISA. At week 7 of the experiment, mice were bled to allow measurements of total and allergen-specific antibodies by ELISA as described (31, 32) with a slight modification. Immunoplates (Nalge Nunc) were coated with 2 μg/ml anti-mouse IgE or anti-mouse IgG antibody (Southern Biotechnology, Birmingham, AL). After washing and blocking of the plates, serial dilutions of serum were added

to the wells, which were then washed. For detection of allergen-specific antibodies, total protein extracts of pollen were biotinilated according to the manufacturer's procedure (Pierce) and added to the wells as a secondary antibody. Total IgE levels in serum were measured by a sandwich ELISA as described (31). After washing the plates, streptavidin–horseradish peroxidase conjugate (Pierce) was added to the wells, and the reaction was developed with peroxidase substrate solution (Moss, Pasadena, MD). The last serum dilution yielding an OD_{450} value of 0.1 over the background was recorded as the endpoint titer for each sample.

T Cell Proliferation and Cytokine Assay. $CD4^+$ T cells were purified from splenocytes at week 7 of the experiment by MACS beads separation using anti-mouse CD4 Ab-conjugated magnetic beads (Miltenyi Biotec). The cells were cultured at 1×10^5 cells per well together with gamma-ray-irradiated splenic antigen-presenting cells at 5×10^5 cells per well for 6 days at 37°C with or without 20 μg/ml total protein extracts of pollen in 96-well plates. Our preliminary study showed that a cedar pollen protein concentration of 20 μg/ml resulted in the optimal dose for the induction of maximum allergen-specific $CD4^+$ T cell responses among the different doses tested (e.g., 4–40 μg/ml). Each well was then pulsed with 0.25 μCi of [^{3}H]thymidine (Amersham Pharmacia; 1 Ci = 37 GBq) for the last 22 h of incubation, and the cells were harvested to allow measurement of radioactivity levels. At the same time, the other aliquots of cells were incubated under identical conditions for 5 days to assess the different Th1- and Th2-type cytokine production by ELISA as described (33).

Histamine Assay. To examine the levels of serum histamine, mice were challenged at week 7 with an i.p. injection of 0.1 mg of total protein extracts of pollen adsorbed on 5 mg of alum. Within 10 min after the injection, blood was taken and histamine levels were determined by using an enzyme immunoassay kit (Neogen, Lexington, KY).

Clinical Symptoms of Pollen Allergy. To examine the effect of A1aB1b-Crp-1 and -2 rice seeds induced oral tolerance for the inhibition of pollen allergen triggered clinical symptoms associating with pollinosis, other sets of mice were fed with the experimental and control rice seeds as described above. These mice were presensitized with pollen allergen via systemic route at weeks 4 and 5. At week 7 through week 8 of the experiment, these mice were then challenged once a day with 20 μl of 1 μg/ml total protein extracts of pollen dissolved in PBS via the intranasal route as described (34). Sham-challenged mice were nasally administered with 20 μl of PBS in the same manner. Nasal symptoms were evaluated by counting the number of sneezes observed in the 5 min after the last nasal challenge at week 8.

Statistics. The significance of the differences (e.g., P values) between groups was evaluated by the Mann–Whitney U test.

Results

Development of Transgenic Rice Plants Accumulating A1aB1b-Crp-1 and -2 Protein in Seeds. Thirty independent transgenic rice plants were generated, and accumulation levels of the A1aB1b-Crp-1 and -2 protein in seeds were examined by immunoblot analysis. Transgenic lines 9 and 12, which showed high levels of accumulation of A1aB1b-Crp-1 and -2 protein at the level of 7 μg per grain ($\approx$0.5% of total seed protein), were selected and proceeded to the T_3 generation by self-crossing to obtain homozygous lines.

To examine the tissue-specific expression of A1aB1b-Crp-1 and -2 gene, total RNA extracted from leaves, roots, and maturing seeds were subjected to Northern blot analysis. The transcript of the A1aB1b-Crp-1 and -2 gene was only detected in maturing seeds, whereas no band was found in the leaves or roots of transgenic lines 9 and 12 (Fig. 1*B*). These results indicate that the A1aB1b-Crp-1 and -2 gene is specifically expressed in seeds under the control of the 2.3-kb *GluB-1* promoter.

Next, total seed protein was extracted for analysis of A1aB1b-Crp-1 and -2 protein expression by Western blot (Fig. 1*C*). We previously demonstrated that the glycinin A1aB1b expressed in the endosperm of transgenic rice was synthesized as a precursor form and then posttranslationally processed into two mature subunits, the acidic and basic subunits (27). As shown in Fig. 1*C*, three signals for the precursor, the acidic and basic subunits with molecular masses of 56, 35, and 21 kDa, respectively, were detected in A1aB1b-Crp-1 and -2 transgenic lines by using anti-glycinin A1aB1b antibody. This result indicates that the A1aB1b-Crp-1 and -2 protein was expressed and posttranslationally processed in a similar manner to the native glycinin A1aB1b (27). The accumulation of Crp-1 and -2 T cell epitope peptides in the A1aB1b-Crp-1 and -2 protein was further confirmed by immunoblot analysis using the peptide specific anti-Crp-1 and anti-Crp-2 antibodies (Fig. 1*C*). It was shown that the glycinin acidic subunit (35 kDa) and the precursor (56 kDa) were recognized by the anti-Crp-1 antibody, whereas the glycinin basic subunit (21 kDa) and the precursor (56 kDa) were detected by the anti-Crp-2 antibody. These results clearly indicated that the Crp-1 and -2 peptides were expressed as fusion protein with A1aB1b and processed into the acidic and basic subunits of A1aB1b, respectively.

Integration of the A1aB1b-Crp-1 and -2 gene into the rice genome was confirmed by Southern blot analysis. Because the SacI restriction enzyme cuts only once in the transformation plasmid, the number of bands indicates the number of copies of the A1aB1b-Crp-1 and -2 gene integrated into the rice genome. At least two copies of A1aB1b-Crp-1 and -2 gene were estimated to be present in transgenic rice lines 9 and 12 (Fig. 1*D*).

Oral Feeding of Transgenic Rice Seeds Prevents the Development of Allergen-Specific IgE and IgG Responses. In the control group of mice fed with PBS, i.p. challenge with the pollen allergen elicited significant allergen-specific IgE and IgG responses (Fig. 2*A* and *C*). Oral feeding of nontransgenic rice seeds did not affect the high levels of allergen-specific IgE antibody response (Fig. 2*A*). In contrast, the level of serum allergen-specific IgE was significantly reduced in the group of mice fed with transgenic rice seeds accumulating A1aB1b-Crp-1 and -2 protein when compared to those in the control groups of mice fed with PBS or nontransgenic rice seeds ($P < 0.01$) (Fig. 2*A*). The levels of total IgE antibodies in serum were similar among the three groups of mice (Fig. 2*B*). In the case of allergen-specific IgG responses, the antibody titers were decreased in mice orally immunized with A1aB1b-Crp-1 and -2 rice seeds when compared to those in the control groups of mice ($P < 0.01$) (Fig. 2*C*). In addition, a dominant allergen-specific IgG1 subclass with some IgG2a and IgG2b antibodies were all decreased in the experimental group of mice (Table 1, which is published as supporting information on the PNAS web site). These results suggest that oral administration of A1aB1b-Crp-1 and -2 seeds inhibits a dominant Th2 cell-mediated antibody with some Th1-involved antibody responses to pollen allergens.

Oral Feeding of Transgenic Rice Seeds Inhibits Allergen-Specific T Cell Proliferation and IgE-Associated Th2 Cytokine Responses. To examine the effect of oral feeding of rice seeds on allergen-specific T cell responses, $CD4^+$ T cells were isolated from the spleens of experimental and control mice and were stimulated *in vitro* with or without pollen allergen. Oral immunization with A1aB1b-Crp-1 and -2 rice seeds greatly suppressed the allergen-specific T cell proliferative responses when compared to those in the control mice (75% suppression, $P < 0.01$) (Fig. 2*D*). To further demonstrate the effect of oral feeding of transgenic seeds on the inhibition of allergen-specific $CD4^+$ T cell responses, we next examined levels of Th1 and Th2 cytokine synthesis (Fig. 3). The amounts of Th1 and Th2 cytokines produced in the culture supernatants of allergen-

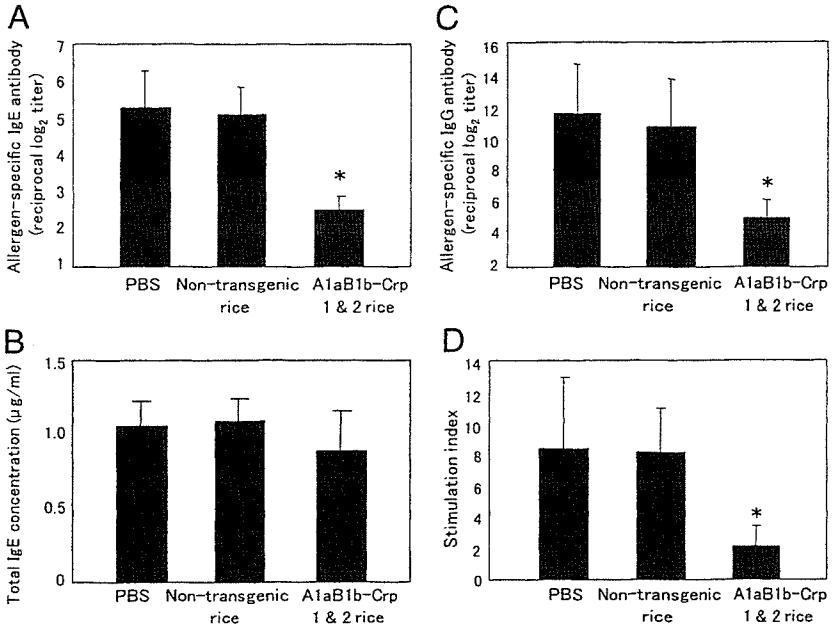

Fig. 2. Inhibition of allergen-specific serum IgE, IgG, and $CD4^+$ T cell responses by oral administration of A1aB1b-Crp-1 and -2 rice seeds. Levels of allergen-specific IgE (*A*), total IgE (*B*), and allergen-specific IgG (*C*) were examined in serum of mice fed with PBS, nontransgenic rice seeds, or A1aB1b-Crp-1 and -2 rice seeds before systemic challenge with total protein extracts of pollen. Allergen-specific splenic $CD4^+$ T responses (*D*) were expressed as stimulation index calculated as the ratio of [cpm of cells cultured in the presence of allergen]/[cpm of cells cultured in the absence of allergen]. Data are expressed as mean ± SD. *, $P < 0.01$ for the group of mice fed with A1aB1b-Crp-1 and -2 rice seeds in comparison with the group of mice fed with PBS or nontransgenic rice seeds.

stimulated $CD4^+$ T cells were measured by ELISA. In the control groups of mice fed with PBS or nontransgenic rice seeds, high quantities of Th2 cytokines associating with IgE-mediated immune responses such as IL-4, IL-5, and IL-13 were produced in the culture supernatants. In the group of mice fed with A1aB1b-Crp-1 and -2 rice seeds, levels of allergic reaction-associated cytokines IL-4, IL-5, and IL-13 were significantly lower than those of control groups of mice (85%, 86%, and 78% suppression, respectively; $P < 0.01$) (Fig. 3). Both Th2-associated IL-10 and Th1-associated IFN-γ cytokines were not induced significantly by this allergic response-inducing system. However, their levels were also decreased in the group of mice fed with A1aB1b-Crp-1 and -2 rice seeds (75% and 59% suppression, respectively; $P < 0.01$) (Fig. 3). The levels of IL-6 were not drastically changed between the three groups. These findings specifically demonstrate that oral immunization of A1aB1b-Crp-1 and -2 rice seeds effectively induced the state of oral tolerance where the inhibition of IgE-associated Th2 cytokines, including IL-4, IL-5, and IL-13, was achieved at the level of allergen-specific $CD4^+$ T cells.

Inhibition of Levels of Histamine Released in Serum of Mice Orally Fed with A1aB1b-Crp-1 and -2 Rice Seeds. Next, we examined the levels of serum histamine release, one of the inflammatory mediators released at degranulation of mast cells associating with IgE-mediated responses (35). Mice were bled within 10 min of the challenge with pollen allergen at week 7 of the experiment. The levels of histamine released in serum were as high as ≈60 ng/ml in mice fed with PBS or nontransgenic rice seeds (Fig. 4*A*). On the other hand, this high level of histamine release was dramatically reduced to ≈20 ng/ml in mice orally immunized with the A1aB1b-Crp-1 and -2 rice seeds (Fig. 4*A*). The results show that oral administration of rice seeds containing A1aB1b-Crp-1 and -2 protein was effective in the induction of oral tolerance for the inhibition of allergy-associated immune responses including those of Th2 cell-mediated IgE response and histamine release.

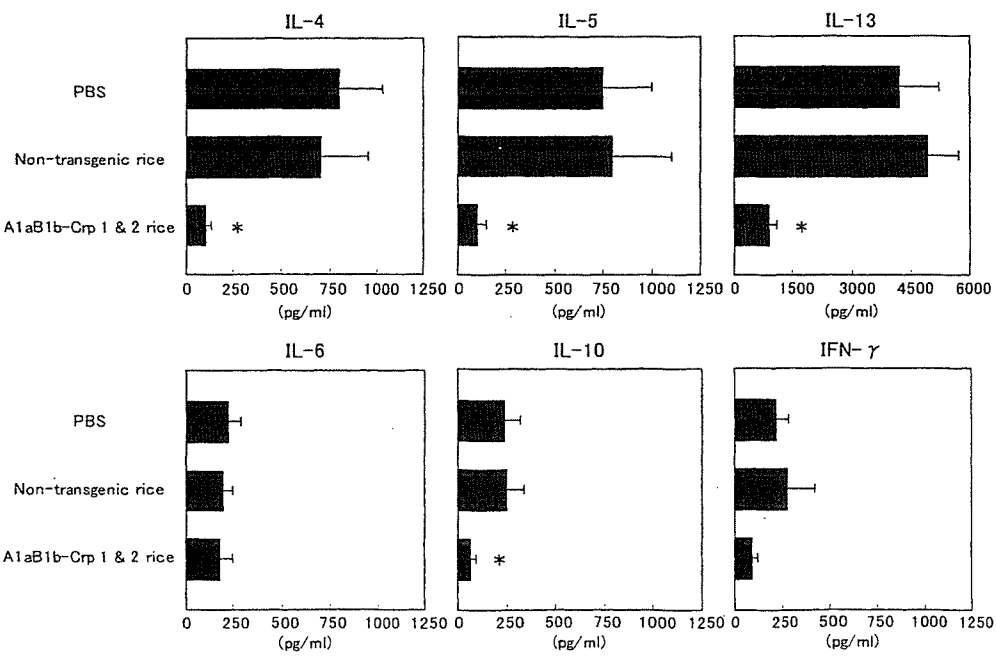

Fig. 3. Inhibition of allergen-induced Th2 cytokine production by splenic $CD4^+$ T cells isolated from mice fed with A1aB1b-Crp-1 and -2 rice seeds. Splenic $CD4^+$ T cells were cultured with or without total protein extracts of pollen as described earlier. Levels of Th1 and Th2 cytokines in cell-free culture supernatants of $CD4^+$ T cells were assayed by ELISA. Data are presented as mean ± SD. *, $P < 0.01$ for the group of mice fed with transgenic rice seeds in comparison with the group of mice fed with PBS or nontransgenic rice seeds.

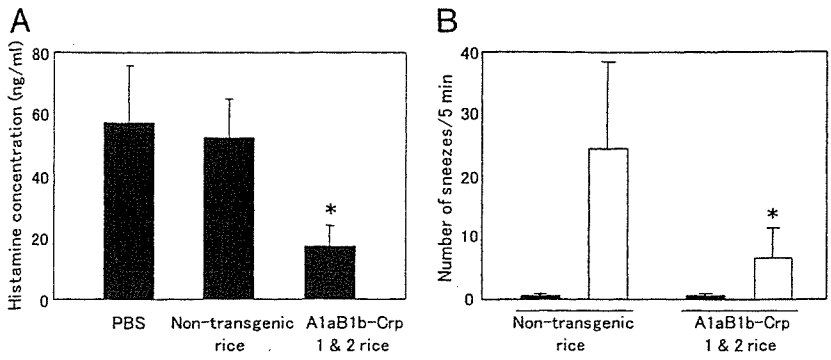

Fig. 4. Serum histamine levels (*A*) and the number of sneezes (*B*) were inhibited in the group of mice fed with A1aB1b-Crp-1 and -2 rice seeds. The number of sneezes was counted in the 5 min after the last nasal challenge at week 8 (white bars). Sham-challenged mice were nasally administered with 20 μl of PBS in the same manner (black bars). Data are expressed as mean ± SD. *, $P < 0.01$ for the group of mice fed with A1aB1b-Crp-1 and -2 seeds in comparison with the group of mice fed with nontransgenic rice seeds.

Inhibition of Pollen-Induced Allergic Symptoms in Mice Orally Fed with A1aB1b-Crp-1 and -2 Rice Seeds. To examine the effect of orally fed A1aB1b-Crp-1 and -2 rice seeds on the development of clinical symptoms of pollinosis, we adopted an experimental murine sneezing model (34). Mice were orally fed with nontransgenic or A1aB1b-Crp-1 and -2 rice seeds and i.p. presensitized with total protein extracts of pollen. Mice were then intranasally challenged with the pollen protein extracts. Significant nasal symptoms of sneezing developed in the group of mice fed with nontransgenic rice seeds (Fig. 4*B*). In contrast, the number of sneezes was reduced in the group of mice fed with A1aB1b-Crp-1 and -2 rice ($P < 0.01$) (Fig. 4*B*). Nasal challenge with PBS did not induce any nasal symptoms of sneezing (Fig. 4*B*). These findings demonstrated that oral administration of A1aB1b-Crp-1 and -2 rice seeds was effective in the induction of tolerance against pollen allergen leading to the inhibition for the development of allergic symptoms of sneezing in nasal tract.

Discussion

Adaptation of the concept of oral tolerance has been considered as a fundamental strategy for the development of immunotherapy for the prevention and/or treatment of allergic diseases (36). The mechanism of oral tolerance has not yet been precisely clarified; however, oral immunization of allergens is known to induce a state of systemic unresponsiveness to the administered allergens (36). To avoid unwanted anaphylactic reactions being elicited during the desensitization process using allergens, the use of T cell epitope peptides has been shown to be an attractive approach (4–8). T cell epitope peptides are incapable of binding to allergen-specific IgE antibody molecules on the surface of mast cells, so the administration of high doses of T cell epitope peptides is theoretically possible without inducing anaphylactic side effects (2). The efficacy of tolerance induction was shown to depend on the dose of allergens administered (7); thus, immunotherapy with T cell epitope peptides is expected to be both safe and effective in the treatment of allergic diseases (2). In this study, we developed transgenic rice plants expressing T cell epitope peptides in seeds and examined whether oral feeding of the transgenic rice seeds to mice could prevent the development of allergic responses against pollen allergens of Japanese cedar. Our results demonstrate that oral immunization of the transgenic rice seeds expressing A1aB1b-Crp-1 and -2 protein resulted in the generation of systemic unresponsiveness with a reduction of allergen-specific Th2-mediated IgE responses and histamine release.

It has been demonstrated that the direct production of short peptides such as T cell epitope peptides with lengths of 10–20 aa is difficult for most expression systems of eukaryotic and prokaryotic cells (37). Therefore, our efforts in this study were initially focused on the expression of Crp-1 and -2 T cell epitope peptides in transgenic rice seeds. We adopted a strategy in which Crp-1 and -2 peptides were expressed as parts of the soybean seed storage protein glycinin by inserting them into highly variable regions of acidic and basic subunits of glycinin A1aB1b (29, 30). The recombinant protein was successfully expressed in rice seeds; however, the maximum level of A1aB1b-Crp-1 and -2 accumulation (0.5% of total seed protein) was lower than that of A1aB1b (5% of total seed protein) (27). One possible explanation for this result is that the insertion of T cell epitopes into variable regions of A1aB1b potentially influences secondary structure formation or interaction between acidic and basic subunits in A1aB1b-Crp-1 and -2, which may cause the lower accumulation levels of A1aB1b-Crp-1 and -2. When expressed in transgenic rice seeds under the control of the glutelin *GluB-1* promoter, glycinin A1aB1b was synthesized as a preproglycinin and posttranslationally processed into acidic and basic subunits (27). The synthesized glycinin A1aB1b was localized in protein body II, in which ≈30% of glycinin was assembled with glutelin (27). In this study, A1aB1b-Crp-1 and -2 protein was synthesized as a precursor form and then posttranslationally processed into acidic and basic subunits in a similar manner to the glycinin A1aB1b (Fig. 1*C*) (27). These results suggest that A1aB1b-Crp-1 and -2 protein accumulated in protein body II, although there is possibility that the insertion of T cell epitope peptides into A1aB1b may affect the intracellular localization of A1aB1b-Crp-1 and -2 protein in the endosperm cells. When anti-Crp-1 peptide antibody was used as a probe, the precursor signal of A1aB1b-Crp-1 and -2 (56 kDa) was weaker than those obtained by the anti-glycinin and anti-Crp 2 peptide antibodies (Fig. 1*C*). These results might be explained by the difference in binding affinity of anti-Crp-1 peptide antibody to the Crp-1 peptide accumulated in two distinct forms, the precursor and mature acidic subunit.

Two regions of pollen allergens (p277–290 of Cry j I and p246–259 of Cry j II) have been identified as major T cell epitopes in BALB/c mice (15, 16). Previously, one of the major T cell epitope peptides, p246–259 of Cry j II, was chemically synthesized and was orally administered to mice before systemic challenge with Cry j II (15). It was shown that the Cry j II-specific IgE response was significantly decreased (74% suppression) in mice orally immunized with the synthetic T cell epitope peptide. Furthermore, both Th1 and Th2 cytokine production was inhibited in the group of mice fed orally with Cry j II peptide compared to the control group of mice fed with PBS (15). In the present study, to evaluate the efficacy of newly generated transgenic rice seeds expressing Cry j I and Cry j II T cell epitope peptides for the induction of systemic unresponsiveness to pollen allergens of Japanese cedar, a group of mice was fed with the transgenic rice seeds and two other groups of mice were orally administered with nontransgenic rice seeds or PBS. We chose total protein extracts of pollen as allergen for the systemic challenge of mice to assess the effectiveness of transgenic rice seeds for taking account of future applications in the clinical treatment of pollen allergy. We further thought that this challenge method has a benefit to examine bystander tolerance effects to additional T cell epitopes. This line of detailed investigation requires further study.

It has been reported that patients with Japanese cedar pollinosis exhibit a high titer allergen-specific IgE response (38). Allergen-specific IgE antibodies have been shown to play a major biological role for the induction of pollen-associated allergic responses (38,

39). In this study, pollen allergen-specific IgE levels were significantly decreased by oral feeding of transgenic rice seeds accumulating A1aB1b-Crp-1 and -2, whereas the levels of total IgE antibodies were similar among the three groups of mice (Fig. 2 *A* and *B*). In addition, oral administration of A1aB1b-Crp-1 and -2 seeds did not affect OVA-specific $CD4^+$ T cell proliferative responses (Fig. 5, which is published as supporting information on the PNAS web site). These results indicate that oral feeding of transgenic rice seeds induces pollen allergen-specific T cell unresponsiveness. Furthermore, it is important to note that glycinin-specific IgG and IgE antibodies were not detected in the sera of control and experimental groups of mice (data not shown).

The production of $CD4^+$ Th2-type cell derived allergen-specific cytokines, IL-4, IL-5, and IL-13, was dramatically inhibited by oral feeding of transgenic rice seeds (Fig. 3). These Th2-type cytokines were shown to be involved in the process of IgE production. IL-4 and IL-13 stimulate and regulate Ig class switching to IgE (39–41) and IL-5 drives the proliferation and differentiation of B cells into antibody-secreting plasma cells (42, 43). The inhibition of these IgE-associated cytokine responses is one of the important factors for the control of allergen-specific IgE synthesis. Our results show that successful inhibition of these cytokine responses offers effective oral immunization by A1aB1b-Crp-1 and -2 rice seeds for the suppression of IgE-mediated hypersensitive allergic reactions. The production of IL-10 was also inhibited in the group of mice fed with transgenic rice seeds, which is consistent with a previous report describing that IL-10 is not required for induction of oral tolerance to OVA (44). On the other hand, it was recently reported that the suppression of allergic diseases in allergen immunotherapy is associated with the increased levels of IL-10 (45, 46). This conflict might be caused by different experimental designs. Further studies are required to examine this controversial result on the role of IL-10 in the induction of oral tolerance.

Allergy-associated inflammatory mediators such as histamine released by mast cells are known to cause immediate symptoms of type-I allergy (35). Allergen-IgE complex formation on the surface of mast cells triggers degranulation of mast cells leading to the histamine release (35). Thus, the reduction of allergen-specific IgE antibody levels can be an effective strategy for the suppression of histamine release by mast cells. In our study, levels of both allergen-specific IgE antibody and serum histamine release were significantly reduced in the group of mice fed orally with A1aB1b-Crp-1 and -2 rice seeds compared with the control groups (Figs. 2*A* and 4*A*). These results suggest that oral immunization of A1aB1b-Crp-1 and -2 rice seeds is effective in the suppression of allergen-specific IgE responses, which further inhibit histamine release by blocking the formation of the allergen-IgE complex. In addition, using the experimental mouse model of pollen allergy, we have shown here that oral feeding of A1aB1b-Crp-1 and -2 rice seeds inhibits the development of nasal allergic symptoms (Fig. 4*B*). Our findings provide further evidence of a significant potential benefit of A1aB1b-Crp-1 and -2 rice seeds for the prevention of the development of IgE-mediated allergic symptoms without any signs of the anaphylactic side effects.

The seed-expression system possesses several advantages for the production of recombinant proteins, such as simplicity of administration, low risk of contamination with animal pathogens, and low cost for production and long storage at room temperature (17–22, 25). Here, we showed that the status of systemic unresponsiveness associated with the inhibition of allergen-specific Th2-type and IgE responses was achieved by oral feeding of recombinant protein containing Crp-1 and -2 T cell epitope peptides without any purification step. Therefore, rice seeds could serve as an effective and new vehicle for the mucosal delivery of pharmatheutical molecules. Further clinical trials will be required to extend our findings for the development of rice-based edible vaccines as a peptide immunotherapy for the control of allergy.

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Enterotoxin-Based Mucosal Adjuvants Alter Antigen Trafficking and Induce Inflammatory Responses in the Nasal Tract

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The safety of nasal vaccines containing enterotoxin-based mucosal adjuvants has not been studied in detail. Previous studies have indicated that native cholera toxin (nCT) can alter antigen trafficking when applied nasally. In this study, we determined the enterotoxin-based variables that alter antigen trafficking. To measure the influence of enterotoxin-based mucosal adjuvants on antigen trafficking in the nasal tract, native and mutant enterotoxins were coadministered with radiolabeled tetanus toxoid (TT). The nCT and heat-labile enterotoxin type 1 (LTh-1) redirected TT into the olfactory neuroepithelium (ON/E). Antigen redirection occurred mainly across the nasal epithelium without subsequent transport along olfactory neurons into the olfactory bulbs (OB). Thus, no significant accumulation of the vaccine antigen TT was observed in the OB when coadministered with nCT. In contrast, neither mutant CT nor mutant LTh-1, which lack ADP-ribosyltransferase activity, redirected TT antigen into the ON/E. Thus, ADP-ribosyltransferase activity was essential for antigen trafficking across the olfactory epithelium. Accumulation of TT in the ON/E was also due to B-subunit binding to GM1 gangliosides, as was demonstrated (i) by redirection of TT by LTh-1 in a dose-dependent manner, (ii) by ganglioside inhibition of the antigen redirection by LTh-1 and nCT, and (iii) by the use of LT-IIb, a toxin that binds to gangliosides other than GM1. Redirection of TT into the ON/E coincided with elevated production of interleukin 6 (IL-6) but not IL-1β or tumor necrosis factor alpha in the nasal mucosa. Thus, redirection of TT is dependent on ADP-ribosyltransferase activity and GM1 binding and is associated with production of the inflammatory cytokine IL-6.

Enterotoxins are powerful mucosal adjuvants; however, the mechanisms for their adjuvanticity are still being defined. Native cholera toxin (nCT) and the *Escherichia coli*-derived heat-labile toxin (human type 1) (nLTh-1) are both potent mucosal adjuvants for coadministered protein antigens when given by the oral, nasal, or parenteral route (4, 9–11, 33, 42, 50). Despite extensive research on these enterotoxins, mucosal adjuvants for human use remain in experimental phases, and recent studies have focused on generating nontoxic mutants of CT (mCT) and LTh-1 (mLTh-1). Detoxification of these enterotoxins was accomplished by site-directed mutagenesis of the ADP-ribosylation site located in the A subunit of these AB_5 enterotoxins (3, 8, 51, 52, 53, 54). These mutants are effective mucosal adjuvants in mice and induce long-term memory for coadministered proteins given either by the nasal or parenteral route (3, 51, 52). In this regard, the nasal route is perhaps superior to oral delivery, since it requires much lower doses of both adjuvant and coadministered proteins/vaccines.

Both nCT and nLTh-1 are part of serogroup I of the heat-labile enterotoxins (38) and display somewhat different ganglioside binding specificities (12). For example, nCT binds predominantly to GM1, while nLTh-1 preferentially binds to GM1 and to a lesser extent to GD1b and binds weakly to GM2 and asialo-GM1 (12). Native LTh-I not only targets gangliosides, but also binds to other glycoproteins in the intestinal tract and is associated with a much larger repertoire of target molecules than has been reported for CT (18, 25). The heat-labile enterotoxins from serogroup II, such as LT-IIb, display different ganglioside binding specificities. LT-IIb binds to GD1a and to a lesser extent to GT1b and showed no affinity for GM1 (12). LT-IIb functioned as a mucosal adjuvant when given nasally and induced a mucosal immune response consistent with a mixed $CD4^+$ Th1/Th2 cell response (34), as was previously reported for nLTh-1 (42). A lack of ganglioside binding, which was accomplished by site-directed mutagenesis of amino acid 33, the G33D mutation, rendered both nCT and nLTh-1 deficient in GM1 binding and in the ability to function as mucosal adjuvants following oral (21) or nasal (7) application. Enterotoxin binding to gangliosides is functionally important for both mucosal adjuvanticity and enterotoxicity. Both nCT and nLTh-1 bind to GM1 on epithelial cells and are endocytosed and transported. Blocking GM1 sites is not sufficient to ameliorate the enterotoxicity of nLTh-1, since the molecule also binds to other intestinal epithelial glycoproteins (26, 55).

ADP-ribosyltransferase activity in nCT may potentially cause damage due to toxicity and inflammation of the nasal epithelium, and in so doing may allow passive entry of codelivered vaccine proteins into the olfactory nerve/epithelium (ON/E) (14). Increased permeability of the gut epithelium for

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low-molecular-weight dextran was seen when nCT was orally administered. This study suggested that increased permeability may be an intricate part of the ability of nCT to function as a mucosal adjuvant (32). This conclusion was supported by the fact that CT-B, which is a poor oral adjuvant, did not cause permeability changes in the gut epithelium (32).

The hypothesis proposed in this study was that part of the adjuvanticity of enterotoxin-based mucosal adjuvants may reflect their ability to alter antigen trafficking in the nasal tract and that this antigen redirection could contribute to enhanced inflammatory reactions, which may differentially boost mucosal immune responses.

In this study, we show that enterotoxin-based mucosal adjuvants, i.e., nCT and nLTh-1, alter codelivered protein vaccine trafficking into the ON/E when given nasally. This process of antigen redirection requires ADP-ribosyltransferase activity of the enterotoxin-based adjuvant, as well as binding to GM1 gangliosides, and coincided with the production of the inflammatory cytokine interleukin 6 (IL-6). On the other hand, mutants of CT and LTh-1 lacking ADP-ribosyltransferase activity did not redirect antigen into the ON/E, nor did the native toxin LT-IIb, which is not able to bind GM1. In conclusion, both ADP-ribosyltransferase activity and GM1 binding are required in order for enterotoxin to redirect antigen into the ON/E.

MATERIALS AND METHODS

Mice. Mice of the C57BL/6 strain 6 to 7 weeks of age were obtained from Charles River Laboratories (Wilmington, MA). The mice were maintained in horizontal laminar flow cabinets and were pathogen free as determined by plasma antibody screening and tissue histopathology performed on sentinel mice. All mice received sterile food and water ad libitum and were between 8 and 12 weeks of age when used for these experiments. All mouse studies were done in accordance with guidelines of both the NIH and the Animal Institutional Care and Use Committee of the University of Alabama at Birmingham to avoid pain and distress.

Enterotoxin production and purification. The enterotoxins were produced in our laboratory, with the exception of nCT, which was purchased (List Biological Laboratories, Inc., Campbell, CA). The mCT (E112K) was generated by site-directed mutagenesis of single-stranded DNA of *Escherichia coli* CJ236 transfected with M13 mp19, which included the CT gene, using the Mutant K system (Takara Biomedicals, Kyoto, Japan) as described previously (29, 53). The glutamate-to-lysine mutation of amino acid 112 was generated using the 5′-GATG AACAAAAAGTTTCTGCT-3′ oligonucleotide (53). The pUC119 plasmid carrying the mutated CT gene was transformed into *E. coli* DH5α. The *E. coli* strains containing the mCT gene were grown in LB broth (10 g NaCl, 10 g tryptone, and 5 g yeast extract/liter) with 100 μg/ml of ampicillin. The resulting mCT, derived from a sonicated cell suspension, was purified by binding to and elution from a D-galactose-immobilized column (Pierce Chemical Co., Rockford, IL).

The enterotoxin gene containing plasmid pMY1900 from *E. coli* strain 1032 was subcloned by PCR into the expression vector pTrc 99A (Amersham Pharmacia Biotech, Piscataway, NJ). The LTh-1 mutant E112K was constructed by site-directed mutagenesis with specific primers as described previously (43, 44). The mLT (E112K) and LTh-1 were purified from sonicated cell suspensions and resuspended in 0.9% NaCl in 10 mM Tris-HCl buffer, pH 8.6. After centrifugation, the supernatant was subjected to a 65% ammonium sulfate precipitation, resuspended in 0.2 M Tris (pH 8.0)–1 M sucrose–10 mM EDTA (TEAN) buffer, and purified on an immobilized D-galactose column (Pierce Chemical Co.) as reported previously (45).

Heat-labile enterotoxin IIb (LT-IIb) was produced with plasmid pTDC101-transformed *E. coli* XL-1 Blue (Stratagene, La Jolla, CA) (5). The recombinant *E. coli* was grown at 37°C with vigorous shaking (225 rpm) in Luria broth (Difco Laboratories, Detroit, MI) supplemented with ampicillin (150 μg/ml; Sigma-Aldrich, St. Louis, MO) in the presence of kanamycin (50 μg/ml; Sigma-Aldrich). The expression of LT-IIb was induced during mid-log phase by the addition of 1 mM isopropyl-β-D-thiogalactoside (Sigma-Aldrich). After 4 h of growth, the bacteria were harvested by centrifugation at 8,000 × *g* for 15 min and resuspended in ice-cold 100 mM Tris-HCl (pH 8.0) containing 20% sucrose, 5 mM EDTA, polymyxin B (100 μg/ml; Sigma-Aldrich), and lysozyme (0.5 mg/ml; Sigma-Aldrich) to release the periplasm content. The supernatants were harvested after 30 min of incubation at 4°C and precipitated by 60% ammonium sulfate saturation. The precipitate was dissolved in 10 mM Tris-HCl (pH 8.0) containing 0.3 M NaCl and filter sterilized prior to gel filtration on a Sephacryl-100 column (Amersham Pharmacia Biotech), followed by chromatography with an anion-exchange Mono Q column (Amersham Pharmacia Biotech). The LT-IIb preparations were analyzed for endotoxin content with the *Limulus* amoebocyte lysate assay kit (BioWhittaker, Inc., Walkersville, MD) using an *E. coli* K235 lipopolysaccharide standard.

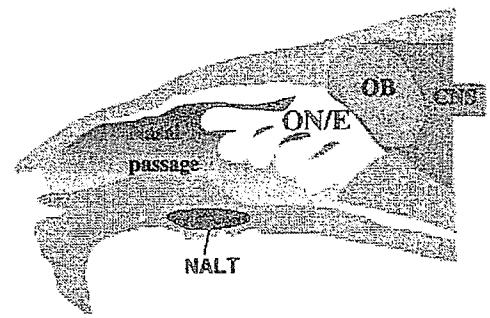

FIG. 1. Anatomy of the murine nasal tract and CNS. Indicated are the locations of the the ON/E, the NALT, and the OB in the nasal tract and adjacent CNS.

Radioiodination of proteins. Tetanus toxoid (TT) (kindly supplied by the Biken Institute, Osaka, Japan) was radiolabeled with ^{125}I. The radioiodination was performed with iodobeads (Pierce Chemicals) for 10 to 12 min at room temperature as described previously (46). Free, unincorporated ^{125}I was removed by dialysis using a Slide Dialyzer (Pierce Chemicals). The trichloroacetic acid-precipitable fraction of ^{125}I-labeled TT was used for all experiments described here. The specific activities of the radiolabeled proteins were 24.5 to 65 cpm/ng. A bicinchoninic acid protein assay (Pierce Chemicals) was used to determine the concentrations of radiolabeled proteins.

Nasal immunization. To assess the ability of ^{125}I-TT to target the ON/E following nasal application, a total of 20 μg of TT (~0.5×10^6 to 1.3×10^6 cpm) was administered in a 10-μl volume, i.e., 5 μl per nare, to naive mice. A total amount of 20 μg of ^{125}I-TT was given either alone or with the indicated enterotoxin delivered in the same volume as antigen alone. For the enterotoxins, we used 1 μg nCT, 10 μg mCT, and 10 μg mLTh-1, and for LTh-1, various amounts of protein were used, i.e., between 1 and 10 μg. For nasal application of the LTII-b enterotoxin, we used 5 μg of protein with ^{125}I-TT.

Trafficking of radiolabeled TT. We used radiolabeled TT protein to track its presence in both lymphoid and central nervous system (CNS) tissues. In these studies, ^{125}I-labeled-TT was given nasally. At 3, 6, 12, 24, and 48 h and 6 days, the ^{125}I-TT levels present in various lymphoid and CNS tissues were determined. For lymphoid tissues, the nasopharyngeal-associated lymphoreticular tissues (NALT), the cervical lymph nodes (CLNs), the mesenteric lymph nodes, the spleen, and blood (50 μl) were assessed. The isolation of NALT was performed as previously reported (49). For the CNS, we examined the ON/E, the olfactory bulbs (OB), and the remainder of the brain. These tissues were isolated as previously described (46). The radiolabeled TT in each tissue was quantitated by use of a gamma counter. The different nasal tract tissues isolated in this study are illustrated in Fig. 1.

In order to assess the influence of blocking the GM1 binding site of LTh-1 or nCT with subsequent tissue distribution of coadministered ^{125}I-TT after nasal application, the LTh-1 and nCT were preincubated with a 15-fold molar excess of GM1 (Sigma-Aldrich) for 30 min at 25°C prior to nasal application. The cpm associated with different tissues 12 h after application were analyzed and compared with application without preincubation with GM1. A total of 20 μg of ^{125}I-TT and 5 μg of LTh-1 or 1 μg nCT either with or without preincubation with free GM1 was nasally administered to individual mice.

Sample collection. Blood was collected into heparinized collection tubes by retro-orbital bleeding of anesthetized mice. The plasma was separated from the cells by a 10-min centrifugation step at 10,000 × *g*. Nasal washes were collected by intubation of the trachea to access the nasopharyngeal cavity. This approach was used to avoid any blood contamination of the nasal washes. A total of 200 μl of phosphate-buffered saline (PBS) was inserted into the nasal cavity, and the

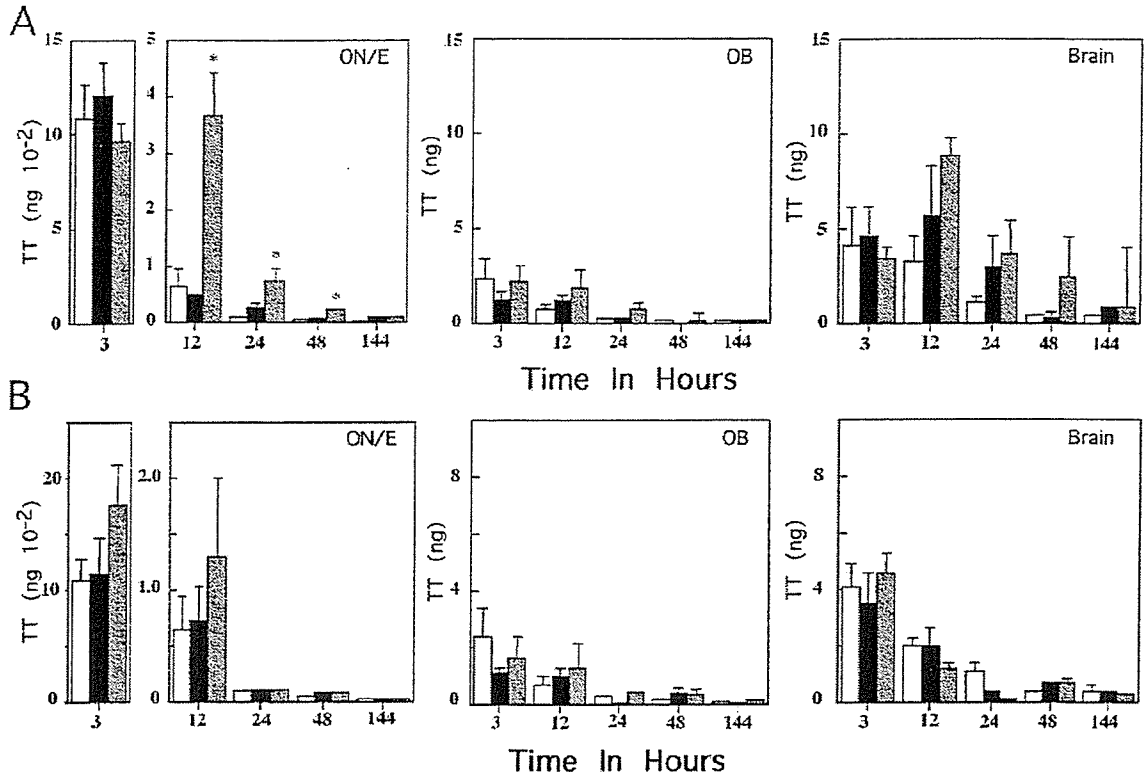

FIG. 2. Comparison of nCT- and mCT- (A) with nLTh-1- and mLTh-1-induced uptake (B) of ^{125}I-TT into olfactory and neuronal tissues. (A) Distribution of ^{125}I-TT in the ON/E, OB, and brain after nasal application of ^{125}I-TT alone (open bars) or in combination with 10 μg of mCT (solid bars) or 1.0 μg nCT (hatched bars) expressed as ^{125}I-TT protein (ng) accumulation. (B) Nasal application of ^{125}I-TT alone (open bars) or in combination with 10 μg of mLTh-1 (solid bars) or 1.0 μg of nLTh-1 (hatched bars) expressed as ^{125}I-TT protein (ng) accumulation. A total of 20 μg of ^{125}I-TT in 12 μl was given nasally either with or without enterotoxin (6 μl/nare). Significant differences between the ^{125}I-TT-only group and ^{125}I-TT-plus-enterotoxin group are indicated by an asterisk and mark P values smaller than 0.05. The averages of 4 to 10 mice plus 1 standard error of the mean are depicted.

exudate from the nares was collected in microcentrifuge tubes. Cells and debris were removed by a 10-min 10,000 × g centrifugation step. All samples were frozen at −80°C until they were analyzed by enzyme-linked immunosorbent assay (ELISA). Lymphoid and neuronal tissues were isolated as described previously (46).

Cytokine ELISA. The detection of the cytokines IL-6, IL-1β, and tumor necrosis factor alpha (TNF-α) in plasma and nasal washes was performed on Maxisorp 96-well immunoplates (Nunc A/S, Roskilde, Denmark). The plates were coated overnight at 4°C with the following capture monoclonal antibodies: rat anti-mouse IL-6 (clone MP5-20F3; BD PharMingen, San Diego, CA), rat anti-mouse IL-1β (clone 30311.1; R&D systems, Minneapolis, MN), and hamster anti-mouse TNF-α (clone TN3-19.12; BD PharMingen) at 2 μg/ml. The plates were washed with PBS-Tween 20 (0.05%) and blocked with 1% bovine serum albumin in PBS-Tween 20 (0.05%) for 1 hour at room temperature. Subsequently, the samples were added to 96-well plates and incubated overnight at 4°C. The plates were washed with PBS-Tween 20, and the biotinylated capture monoclonal antibodies rat anti-mouse IL-6 (clone MP5-32C11; BD PharMingen), goat anti-mouse IL-1β (R&D Systems), and rabbit anti-mouse TNF-α (BD PharMingen) at concentrations of 0.5 μg/ml, 300 ng/ml, and 0.5 μg/ml, respectively. For detection of IL-1β and TNF-α, streptavidin-conjugated to horseradish peroxidase (Life Technologies Inc., Rockville, MD) was used at a 1:2,000 dilution and anti-biotin-horseradish peroxidase at a 1:2,000 dilution (Vector Laboratories, Burlingame, CA) was used for IL-6. The ELISA plates were washed, followed by a 15-min incubation with 2,2′-azino-bis-(3)-ethylbenzylthiazoline-6-sulfonic acid substrate (Sigma Chemical Co., St. Louis, MO). The absorption at 415 nm was measured at various sample dilutions, and the cytokine levels were determined using standard curves. The detection limits of the ELISA for IL-6, IL-1β, and TNF-α were 10, 1, and 12 pg/ml, respectively.

Statistics. The data are expressed as the mean plus 1 standard error of the mean, and the results were compared by the two-tailed, unpaired Mann-Whitney or Student t test. The results were analyzed using the Statview II statistical program (Abacus Concepts, Berkeley, CA) adapted for MacIntosh computers.

RESULTS

Redirection of TT into the ON/E. In order to define the parameters involved in redirection of vaccine proteins into olfactory tissues by nCT and nLTh-1, the role of ADP-ribosyltransferase activity in antigen redistribution was first analyzed by comparison with nonenterotoxigenic mutants of CT (E112K) and LTh-1 (E112K). The presence of ^{125}I-TT in the ON/E, OB, and brain was assessed after nasal application of ^{125}I-TT only or in combination with nCT or mCT (E112K) (Fig. 2A) or with nLTh-1 or mLTh-1 (E112K) (Fig. 2B). The enterotoxin dose was based on the amount required to induce strong mucosal immune responses after nasal application. No significant differences were observed between the distribution of TT protein when given alone or with nCT (1 μg), mCT (10 μg), nLTh-1 (1 μg), or mLTh-1 (10 μg) in all tissues tested, with the exception of the ON/E. Strikingly elevated levels of TT protein were present at 12, 24, and 48 h in the ON/E (P = 0.004, P = 0.028, and P = 0.043, respectively) when given

nasally with nCT. However, minimal uptake was seen at these time points when TT was given alone or with mCT, nLTh-1 (1 μg), or mLTh-1 (Fig. 2A and B). Although the elevated redistribution of ^{125}I-TT into the ON/E induced by nCT was reproducible, it is unlikely that the 48-h time point was of biological importance, since the differences between the groups were relatively small. The nCT-versus-mCT results clearly show that ADP-ribosyltransferase activity is required for redirection of TT into the ON/E. However, it was noteworthy that nLTh-1 (1.0 μg) did not induce significant increases of TT protein in the ON/E at 12 h following nasal application. This TT accumulation was considerably lower than that seen with 1.0 μg of nCT and indicated that factors in addition to ADP-ribosyltransferase activity also play roles in protein redistribution. It should be noted that no preferential accumulation of TT was observed in the OB versus the brain as previously reported for CT-B and CT (46). Thus, unlike CT or CT-B, limited or no axonal transport of TT along olfactory neurons takes place after nasal application, and the distribution of TT in the OB and brain can be explained by the sizes of the organs and the blood associated with them.

Since nCT and nLTh-1 display different ganglioside binding specificities, where nLTh-1 binds in addition to GM1, as reported for nCT and also GD1b, GM2, asialo-GM1, and other intestinal glycoproteins (12, 18, 25), it remained possible that GM1 binding by the enterotoxin was required for protein redirection into the ON/E. Thus, decreased binding by nLTh-1 to GM1 may be due to competition by other nLTh-1 receptors in the nasal tract, which would inhibit protein redirection. In addition, it should be noted that the uptake of TT into various tissues, when 1 μg nLTh-1 was used, was about half of the total uptake observed when 1 μg of nCT was given nasally. To test the GM1 dependence of TT redirection, a dose-response experiment with nLTh-1 was performed (Fig. 3A). Increasing levels of nLTh-1 induced enhanced TT redirection, and this required between a two- and fivefold-higher dose to induce levels not significantly different from those seen with 1 μg of nCT. This observation is consistent with a requirement to target GM1 in order to redirect protein into the ON/E. A comparison between the abilities of nCT, mCT, nLTh-1, and mLTh-1 to redirect protein into ON/E was made (Fig. 3B). In conclusion, nCT was more potent than nLTh-1 in redirecting codelivered protein into the ON/E, while the nontoxic mCT or mLTh-1 was only marginally able to do so at the levels tested.

Distribution of TT in lymphoid tissues. After nasal application of ^{125}I-TT, the NALT, blood, spleen, and CLNs were isolated and analyzed for the distribution of protein when given alone or in combination with nCT, nLTh-1, mCT (E112K), and mLTh-1 (E112K). No significant differences were seen in these lymphoid tissues with the exception of NALT. A significantly decreased accumulation of TT was seen in NALT of mice given TT nasally with nCT (Fig. 4). A small decrease in TT protein accumulation in NALT was observed with mCT, and no significant differences were seen between nLTh-1 and mLTh-1 compared with TT given alone. The data from these observations are summarized in Table 1 and were compared with the observed immune responses induced by these enterotoxin-based mucosal adjuvants. It was interesting that only decreased antigen accumulation in NALT was observed when a strong Th2 response was induced.

The role of gangliosides in protein redirection. In order to assess the role of GM1 ganglioside binding by nLTh-1 and nCT for ^{125}I-TT redirection into the ON/E, the ganglioside binding site was blocked by prior incubation with a 15-fold molar excess of GM1. Blocking the ganglioside binding site of nLTh-1 and nCT with GM1 significantly inhibited redirection of ^{125}I-TT into the ON/E for both 5 μg of nLTh-1 ($P = 0.04$) and 1.0 μg of nCT ($P = 0.02$) 12 h after application (Fig. 5). Blocking the ganglioside binding site also elevated TT levels in the blood, spleen, and CLNs. A significant increase in TT accumulation was observed in the CLNs (which drain the nasal tract) 12 h after application with nLTh-1 (data not shown). Whether this increase of protein in the CLNs was due to a lack of ON/E targeting and resulted in subsequent drainage into the CLNs or was due to enhanced circulation in the blood, or a combination of the two, cannot be distinguished.

In order to determine whether binding to gangliosides other than GM1 would prevent antigen redirection into the ON/E, the heat-labile enterotoxin from serogroup two, LT-IIb, was used. This enterotoxin displays high-affinity binding to GD1a and GT1b and weak affinity for GM3 and does not bind at all to GM1 (12). Furthermore, LT-IIb is more toxic to Y1 adrenal cells than nCT based upon morphological changes and adenylate cyclase activation (24). When the ability of the LT-IIb enterotoxin was compared with that of nLTh-I for redirection of TT into the ON/E, it was very apparent that 5 μg of LT-IIb, unlike nLTh-I, was unable to redirect ^{125}I-TT into the ON/E and did not affect the TT distribution pattern observed in other tissues (Fig. 6). Thus, GM1 targeting appears to be an essential step in directing the ADP-ribosyltranferase to cause codelivered antigen redirection. As pointed out in Fig. 2, the TT associated with the OB was considerably lower than that observed in the brain and reflected the smaller size and lower amount of blood associated with these organs and argues against considerable axonal transport of TT from the nasal tract into the OB as observed with labeled CT and CT-B (46).

Differential production of inflammatory cytokines in the nasal tract. To determine if nasal application of mucosal adjuvants induces inflammatory cytokines, nasal washes and plasma were collected at various time points after nasal delivery. The nasal washes and plasma were analyzed for IL-1β, IL-6, and TNF-α. Differential expression of IL-6 was seen in the nasal washes (Fig. 7). Both nCT- and mCT-treated mice displayed IL-6 levels significantly elevated over those seen when TT was given alone (Fig. 7). Although the levels of IL-6 at 6 h were twofold higher in the nCT- than in the mCT-treated mice, no significant differences were seen between these groups until 12 h after application ($P = 0.026$). Markedly lower levels of IL-6 and IL-1β were seen in the plasma of the mice. The time frame between 3 and 12 h following administration of nCT and TT, when redirection of TT into the ON/E was observed (Fig. 2A), also represented the time when maximal IL-6 secretion was noted in nasal washes. Thus, local inflammatory responses were induced by nCT, and to a much lesser extent by mCT, during this time period. No detectable levels of TNF-α were observed in either plasma or nasal washes (data not shown), and IL-1β levels did not differ significantly among the three groups. These differences in production of IL-6 were not due to the differences in lipopolysaccharide, since the nCT contained ≤0.048 ng/μg and the mCT contained ≤1.0 ng/10

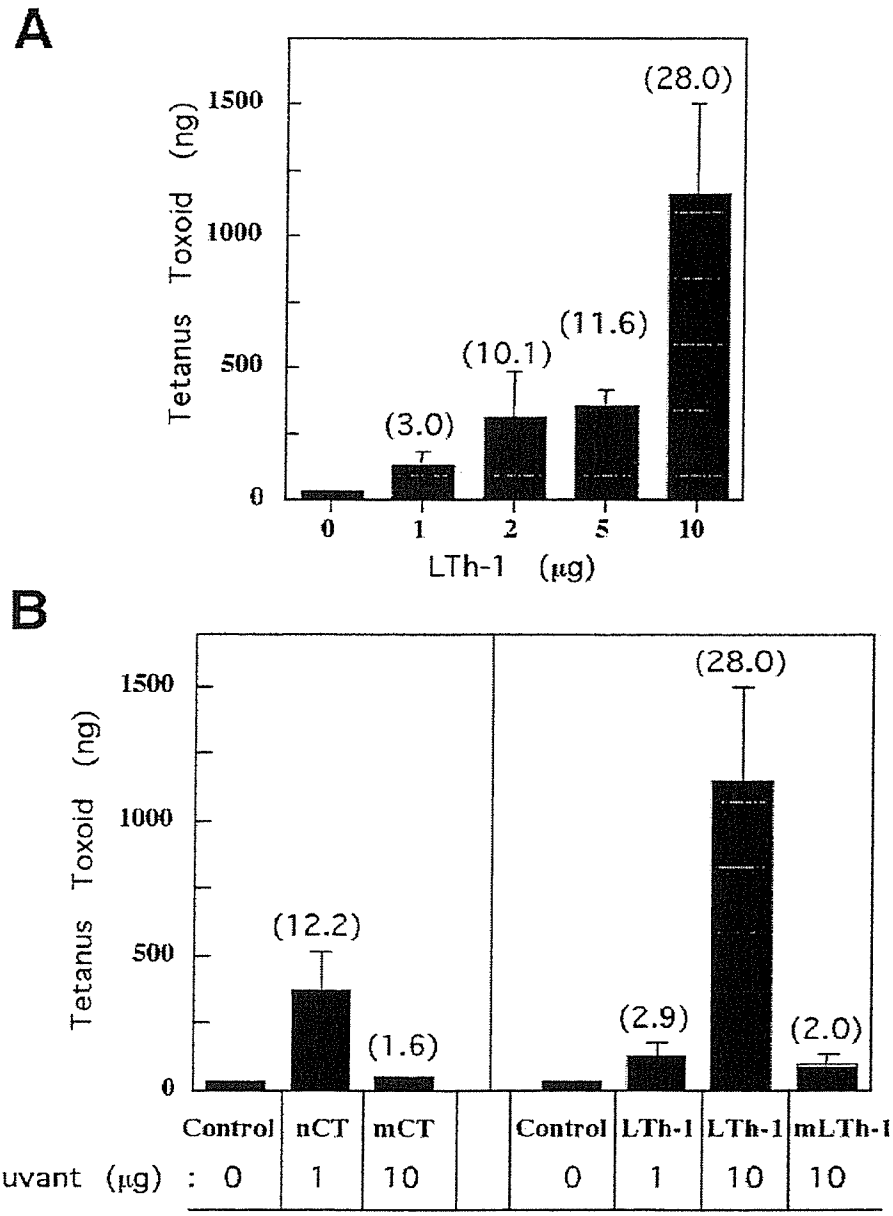

FIG. 3. Enterotoxin-dependent redirection of ^{125}I-TT into the ON/E. Various doses of nLTh-1, i.e., 0, 1, 2, 5, and 10 μg, were combined with 20 μg ^{125}I-TT and applied nasally. The ON/E were collected 12 h after application to assess redirection of the protein. (A) Indicated is the increase (*n*-fold) over control values, i.e., ^{125}I-TT alone, when administered with nLTh-I. (B) Comparison of the degrees of redirection of ^{125}I-TT into ON/E observed with nCT, mCT (E112K), nLTh-1, and mLTh-1 (E112K). Indicated are the means plus standard errors of the mean.

μg. The observations for IL-6 were confirmed by real-time reverse transcription-PCR on RNA derived from the ON/E (data not shown).

To determine whether LTh-1 had similar effects on IL-1β and IL-6 production, nasal washes and plasma were collected from mice treated 12 h prior with TT alone or TT with 1.0 or 5.0 μg of LTh-1. A significant increase in IL-6 was seen in nasal washes when 5 μg of LTh-1 was given with TT, while no significant increase was observed in plasma (Fig. 8). Elevated secretion of IL-6 was also seen in nasal washes with the 1.0-μg LTh-1 dose; however, this increase in IL-6 was not significant compared with TT alone.

DISCUSSION

The redirection of TT protein by native enterotoxin-based mucosal adjuvants raises questions regarding both safety and the molecular mechanisms involved. In this study, we addressed the parameters influencing redirection of the vaccine protein TT into the olfactory nerve/epithelium, NALT, and related lymphoid tissues, as well as the associated production of inflammatory cytokines in the nasal tract. To redirect nasally coadministered ^{125}I-TT into the ON/E by enterotoxin-based mucosal adjuvants, ADP-ribosyltransferase activity is clearly required. This is based upon the finding that both nCT and

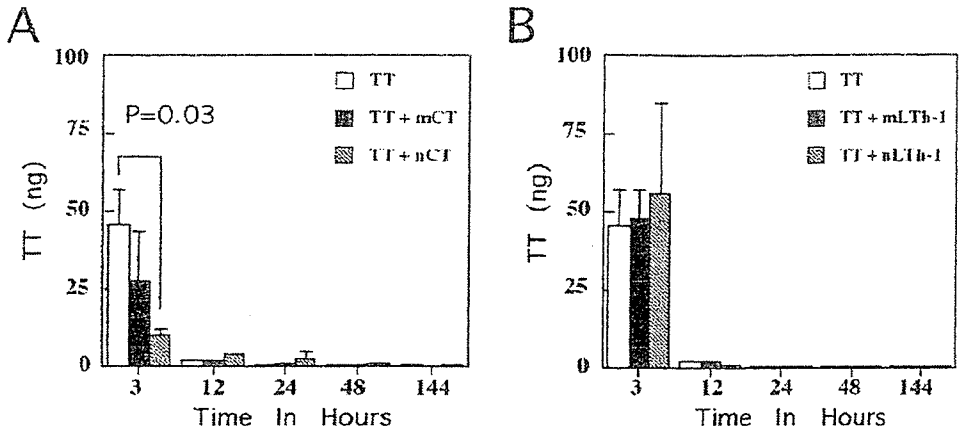

FIG. 4. Comparison of nCT- and nLTh-1- with mCT (E112K)- and mLTh-1 (E112K)-induced uptake of ^{125}I-TT into NALT. Distribution of ^{125}I-TT in NALT is expressed as TT (ng) accumulation per organ. A total of 20 μg of ^{125}I-TT alone or in combination with 10 μg of mCT or 1.0 μg of nCT (A) or ^{125}I-TT alone or in combination with 10 μg of mLTh-1 or 1.0 μg nLTh-1 (B) was given nasally (6 μl/nare). Accumulation of ^{125}I-TT was analyzed at various time points after application. The average of 5 to 10 mice plus standard error of the mean per data point are depicted.

nLTh-1 redirected protein into the ON/E while mCT (E112K) and mLTh-1 (E112K), which both lack ADP-ribosyltransferase activity (28, 54), did not. Thus, ADP-ribosyltransferase activity of the A subunit is an essential, although not sufficient, element for redirection of protein into the ON/E by AB_5 enterotoxins. Binding to GM1 by the B subunit, in addition to ADP-ribosyltransferase activity of the A subunit, also appears to be a prerequisite for redirection of protein into the ON/E, since incubation of nCT or nLTh-1 with excess GM1 prior to administration prevented accumulation of ^{125}I-TT in the ON/E.

The finding that LT-IIb does not redirect TT into the ON/E while it retains full ADP-ribosyltransferase activity and functions as a mucosal adjuvant when delivered nasally (34) may be explained by a requirement for GM1 binding by the enterotoxin to induce protein redirection, since LT-IIb, unlike nCT or nLTh-1, does not bind to GM1 gangliosides (12, 38). Using a human intestinal epithelial cell line (T84), others found that both nCT and LT-IIb bound with high affinity (2 to 5 μM) to the apical membranes of T84 cells (48). However, only nCT was able to elicit a cyclic-AMP-dependent secretory response. Moreover, while nCT-GM1 fractionated with a caveola-like, detergent-insoluble membrane fraction, the LT-IIb-GD1a complex was solubilized by 1% Triton X-100. The authors suggested that signal transduction may require the formation of caveola-like structures and demonstrated that the chimera composed of the LT-IIb A subunit and CT-B was capable of inducing a secretory response. Native CT binding to polarized epithelial cells takes place on the apical membrane surface but targets a basolaterally located effector molecule, i.e., adenylate cyclase (31). It could be hypothesized that the requirement to bind GM1 is associated with the endocytotic pathway taken following GM1 endocytosis. The LT-IIb binds to GD1a, a ganglioside that is not located in the caveola-like membrane domains as reported for GM1 (48), and may thus follow a different intracellular path in epithelial cells. As a consequence of this, the A1 subunits or the ADP-ribose-Gsα may not reach the adenylate cyclase located in the basolateral domain of polarized epithelial cells. This could be an important step for enhanced permeability of the epithelium and the ability of antigen to cross the nasal epithelial barrier.

The nasal tract is covered by a pseudostratified epithelium. Underneath this epithelium, a dense network of fenestrated capillaries provides a readily available blood supply (14). The nasal administration of enterotoxin-based adjuvants would target this epithelium through GM1 ganglioside binding. Our observations and those of others reporting the use of rabbit mucosa in vitro (14) clearly raise questions regarding the safety of nasal use of these adjuvants in humans. Human studies performed previously using nasal nLTh-1 and CT-B suggest that precautions need to be taken when applying these mole-

TABLE 1. Antigen-specific immune response and antigen distribution in NALT following nasal immunization

Adjuvant	Antigen	Plasma IgG[a]	S-IgA[b]	T helper activity	TT in NALT[c]	Reference(s)
	TT	±[d]	+	Th1/Th2	++++	30, 47, 51
nCT	TT	±+++	+±+	Th2	+	47, 50, 51
mCT(E112K)	TT	±+++	+±+	Th2	++	50, 51
nLTh-1	TT	±+++	+±+	Th1/Th2	++++	2, 7, 42
mLTh-1(E112K)	TT	±+++	+±+	Th1/Th2	++++	2, 7
LTIIb	AgI/II[e]	±+++	+±+	Th1/Th2	++++	34

[a] IgG, immunoglobulin G.
[b] S-IgA, mucosally-derived secretory IgA.
[c] Data were obtained in this study and indicate the relative TT antigen accumulation in NALT.
[d] ±, low-; ++, medium-; +++, high-; ++++, very high.
[e] AgI/II, Antigen I/II from *Streptococcus mutans*.

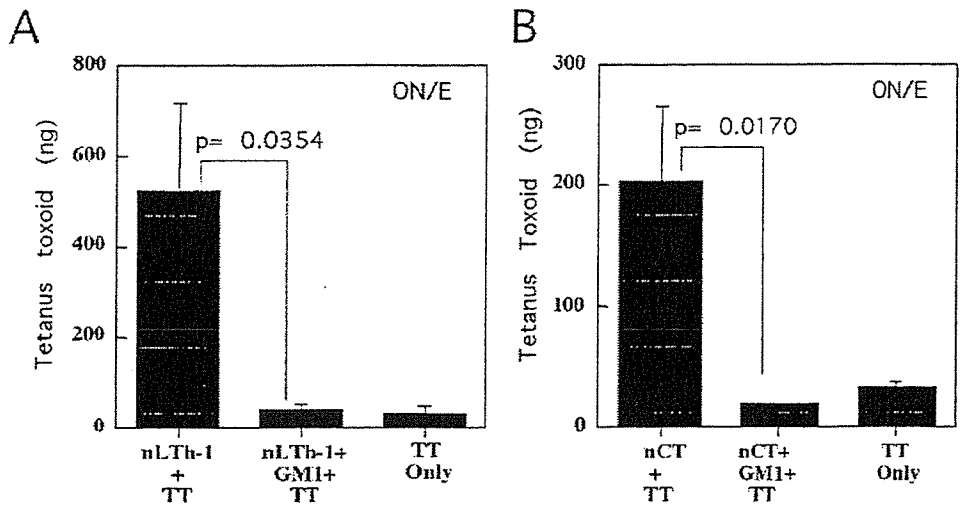

FIG. 5. Influence of blocking the GM1 binding site on nLTh-1 and nCT on tissue distribution of coadministered ^{125}I-TT in the ON/E after nasal application. nLTh-1 (A) and nCT (B) were preincubated with a 15-fold molar excess of GM1 for 30 min at room temperature prior to nasal application together with ^{125}I-TT. The cpm associated with the ON/E 12 h after application were analyzed and compared with application without preincubation with GM1 and with administration of ^{125}I-TT antigen alone. A total of 20 μg ^{125}I-TT with 5 μg of nLTh-1 or 1.0 μg of nCT was nasally delivered to individual mice. The results are from five mice per group. Indicated are the means plus standard errors of the mean.

cules to the human nasal tract. Human studies involving nasal application of CT-B reported mild adverse effects, which resolved within 24 h (1, 39–41). The tolerated and effective dose was between 100 and 500 μg for nasally applied CT-B in a nasal spray/aerosol. At the lower end of an effective immunization range, i.e., 100 μg of CT-B, 7 out of 20 patients and all in the high range (the 1,000-μg group), experienced adverse effects. The symptoms consisted of profuse nasal secretions, itching, and violent sneezing, which resolved within 1 day (1). Although the use of a high-dose CT-B is different from our toxin-mediated antigen redirection, it could be argued that high doses of CT-B will accumulate in the olfactory neuroepithelium, as has been reported for mice (46), and consequently would lead to induction of proinflammatory cytokines throughout the nasal tract.

The results with nasally applied CT-B in humans are consistent with our observations that high levels of IL-6, and to a lesser extent IL-1β, are present in the nasal tract during the first 24 h after application. Interleukin 6 was expressed during the peak of TT protein redirection into the ON/E, and at 12 h was significantly higher in nCT-treated mice than in mice treated with mCT E112K. Furthermore, both enterotoxins induced significantly higher IL-6 levels in nasal washes than were seen in mice given TT only. IL-6 is a multifunctional cytokine that influences both innate immune reactions, such as inflammation (36) and acute-phase responses, and specific immunity, such as B-cell differentiation. IL-6 is produced by a variety of cells, including epithelial cells, macrophages, fibroblasts, and T cells. Native CT rapidly induces IL-6 secretion by the rat intestinal epithelial cell line IEC-6 (35). Whether IL-6 plays a

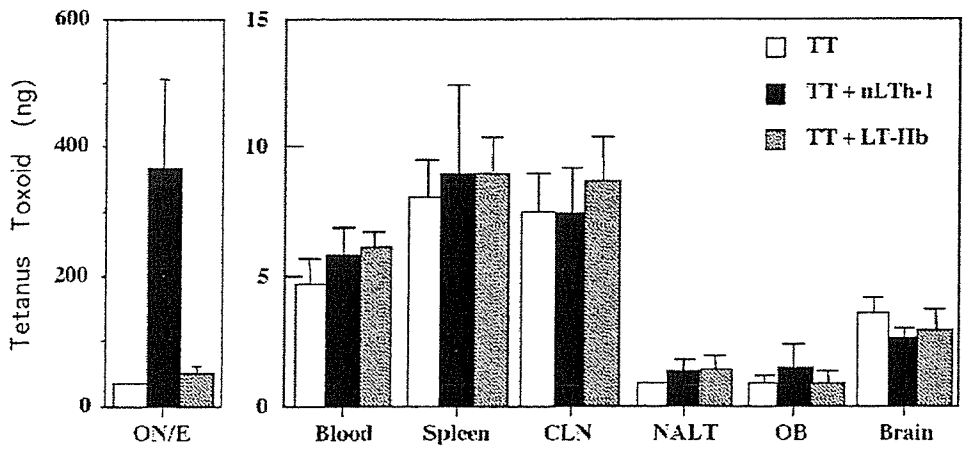

FIG. 6. Trafficking of ^{125}I-TT (20 μg) given nasally without or with nLTh-1 (5 μg) or LT-IIb (5 μg) as mucosal adjuvant. The uptake of ^{125}I-TT into the ON/E, OB, brain, NALT, CLNs, blood, and spleen is shown 12 h after nasal application. The results depicted are from five mice/group and are representative of three separate experiments. Indicated are the means plus standard errors of the mean.

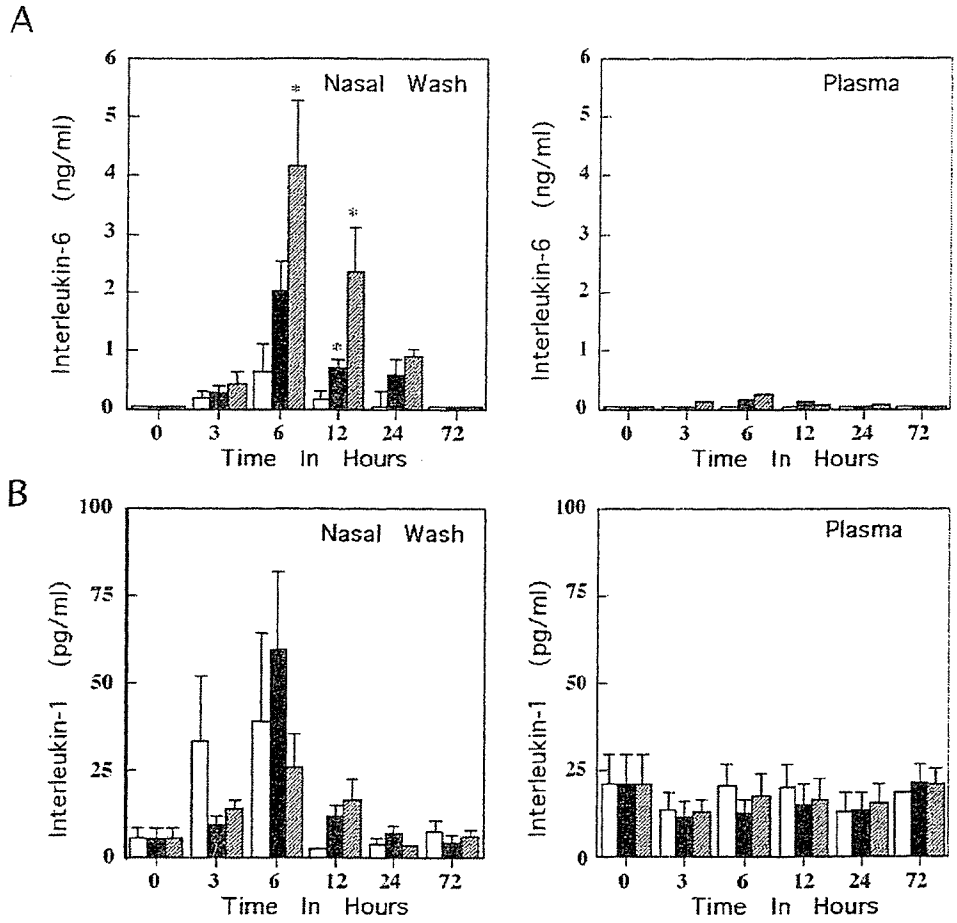

FIG. 7. Inflammatory cytokine expression in the nasal tract after nasal application of TT with or without mCT or nCT. Nasal washes were collected at 0, 3, 6, 12, and 24 h after nasal application of TT alone (open bars), TT and mCT (solid bars), or TT and nCT (hatched bars). The inflammatory cytokine levels for IL-6 (A), IL-1β (B), and TNF-α were determined by ELISA. No TNF-α was detected in the nasal washes. Indicated are the means plus standard errors of the mean for IL-6 and IL-1β. The asterisks indicate significantly elevated cytokine levels ($P < 0.05$) when nCT or mCT with TT was compared with TT alone. The results are representative of two separate experiments.

role in antigen redirection remains to be determined; however, it is more likely that multiple factors contribute to antigen redirection into the ON/E. Specifically, neuropeptides could be major players in this process. For example, vasoactive intestinal peptide (VIP) plays an important role in fluid accumulation in the rat jejunum following stimulation with nCT or nLTh-1 (27). Furthermore, CT-B seems to specifically target VIP-containing neurons in the intestinal tract (15). These observations indicate that VIP could also be important for nasal reactogenicity and antigen redirection.

Human studies involving nasal application of CT-B have focused on the induced immune responses to CT-B rather than on its properties as a mucosal adjuvant (1, 39–41). However, the adjuvant properties of nLTh-1 were assessed in humans given two nasal applications a week apart with an aerosolized virosome-formulated influenza vaccine containing 1.0 μg or 2.0 μg of nLTh-1 for induction of influenza virus-specific immune responses (16, 17). The nLTh-1 functioned as a mucosal adjuvant in humans and induced influenza virus-specific immune responses; however, about 50% of the subjects experienced some type of local or systemic adverse reaction. These reactions included rhinorrhea, stuffiness, sneezing, and headaches, but most of them were mild and resolved within 48 h (16). Similar reactions have also been reported with nasal influenza virus vaccine given with 99.5 μg LT-B and 0.5 μg nLTh-1 (23). The results are consistent with our observations with nCT and nLTh-1 in that redirection of coadministered antigen into the ON/E and production of inflammatory cytokines resolved within 48 h, indicating that similar events could have taken place in humans.

Despite the similarities between mice and humans, the relative surfaces of the nasal tract that constitute the olfactory epithelium are quite different. In mice, approximately 45% of the nasal tract surface constitutes olfactory epithelium (19), while in humans it is an estimated 2.5 cm^2 which would translate to ~2 to 3% of the nasal surface (13, 22). Thus, in mice, nasal application is probably more likely to target olfactory neurons than in humans. Furthermore, the nasopharynx has a 90° angle in humans while there is only a 15° angle in mice. Due to the much larger volume of the human nasopharynx (20 ml) than the mouse nasopharynx (30 μl) and the larger angle, it is likely much harder to consistently target the olfactory

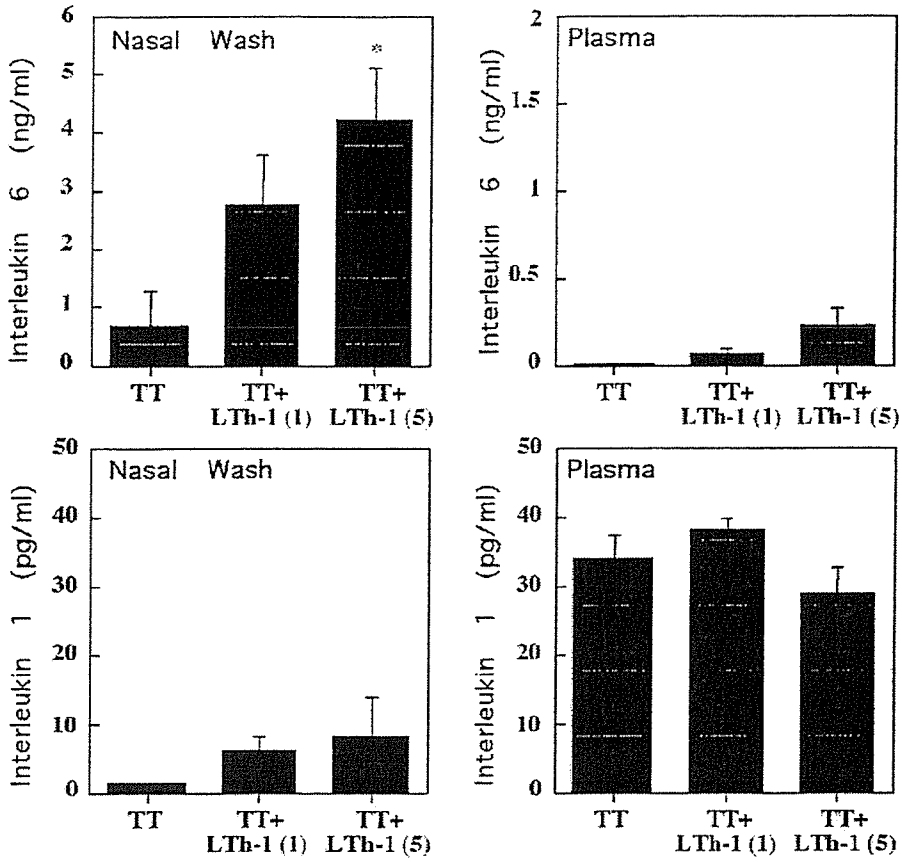

FIG. 8. Inflammatory cytokine expression in the nasal tract after nasal application of TT with or without LTh-1. Nasal washes and plasma were collected 12 h after nasal application of TT (10 μg) alone, TT and LTh-1 (1) (1.0 μg), or TT and LTh-1 (5) (5.0 μg). The levels of IL-6 and IL-1β in nasal washes and plasma were measured by ELISA. Indicated are the means plus standard errors of the mean for IL-6 and IL-1β of five mice per group. The asterisk indicates significantly elevated cytokine levels ($P < 0.05$) when LTh-1 given with TT was compared with TT given alone. The results are representative of three separate experiments.

epithelium in humans using nasal drops, while in mice this would be very reproducible (13). It could be argued that because of the above-outlined reasons nasal sprays would more consistently target the olfactory epithelium in humans than nasal drops.

The observation that nCT significantly reduces TT accumulation in NALT 3 h after nasal application compared to TT alone or TT plus nLTh-1 (Fig. 4) is interesting from the perspective that exposure to a low dose of soluble protein is associated with induction of a Th2-type T helper cell response (6, 20, 37, 47). The induction of potent Th2-type helper activity specific for antigens codelivered with nCT (33, 50) or mCT (30, 51, 53, 54) and the induction of a mixed Th1/Th2 response to antigen coadministered with nLTh-1 (2, 7, 42), mLTh-1 (2, 7), or LTIIb (34) coincide with decreased antigen accumulation in NALT with a strong Th2 response but not with the mixed Th1/Th2 response (Table 1). For example, antigen accumulation in NALT is approximately sixfold lower with nCT than with nLTh-1. It will be interesting to see in future studies whether this altered antigen level will translate into an altered cytokine environment in the NALT for induction of a TT-specific immune response.

In summary, the redirection of a vaccine protein into the olfactory tissues by enterotoxin-based mucosal adjuvants following nasal administration is associated with reactogenicity in the nasal mucosa. The differential accumulation of TT protein in NALT when administered with nCT or nLTh-1 may have consequences for the induced TT-specific T helper cell responses. The parameters controlling antigen redirection into the ON/E include ADP-ribosyltransferase activity of the A subunit and GM1 ganglioside binding by the B subunit. Thus, redirection of vaccine antigen into the ON/E by enterotoxin-based mucosal adjuvants, such as nCT and nLTh-1, clearly requires both ADP-ribosyltransferase activity and targeting of GM1 gangliosides.

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nature immunology

Essential function for the kinase TAK1 in innate and adaptive immune responses

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Transforming growth factor-β–activated kinase 1 (TAK1) has been linked to interleukin 1 receptor and tumor necrosis factor receptor signaling. Here we generated mouse strains with conditional expression of a *Map3k7* allele encoding part of TAK1. TAK1-deficient embryonic fibroblasts demonstrated loss of responses to interleukin 1β and tumor necrosis factor. Studies of mice with B cell–specific TAK1 deficiency showed that TAK1 was indispensable for cellular responses to Toll-like receptor ligands, CD40 and B cell receptor crosslinking. In addition, antigen-induced immune responses were considerably impaired in mice with B cell–specific TAK1 deficiency. TAK1-deficient cells failed to activate transcription factor NF-κB and mitogen-activated protein kinases in response to interleukin 1β, tumor necrosis factor and Toll-like receptor ligands. However, TAK1-deficient B cells were able to activate NF-κB but not the kinase Jnk in response to B cell receptor stimulation. These results collectively indicate that TAK1 is key in the cellular response to a variety of stimuli.

Proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin 1β (IL-1β) have a critical function in innate immune responses by eliciting inflammation[1,2]. The production of proinflammatory cytokines can be induced by various cellular stresses, including pathogenic infection. The initial recognition of invading pathogens is mediated by Toll-like receptors (TLRs), which detect distinct pathogen-associated molecular patterns[2–4]. Stimulation of cells with TLR ligands, IL-1β and TNF activates intracellular signaling pathways leading to the activation of transcription factors such as NF-κB and AP-1 (ref. 1). Activation of AP-1 is mediated by mitogen-activated protein kinases (MAPKs), including Erk, Jnk and p38. Ultimately, these transcription factors initiate expression of genes involved in inflammatory responses. It is well known that TLRs and IL-1 receptor (IL-1R) activate similar signaling pathways[3]. The cytoplasmic portions of TLRs and IL-1Rs contain the Toll–IL-1R homology domain. Ligand stimulation recruits MyD88, a Toll–IL-1R homology domain–containing adaptor protein, to the Toll–IL-1R homology domain of the receptor. Subsequently, IL-1R-associated kinases (IRAKs) are recruited and phosphorylated, and then they interact with TNF receptor (TNFR)–associated factor 6 (TRAF6)[3]. TRAF6 comprises an N-terminal RING finger domain, which has been found in a family of E3 ubiquitin ligases[5]. It has been proposed that a dimeric ubiquitin-conjugating enzyme complex composed of Ubc13 and Uev1A, together with TRAF6, can catalyze the formation of a K63-linked polyubiquitin chain[5,6]. The ubiquitination is responsible for the activation of IκB kinases (IKKs). Subsequently, phosphorylated IκB undergoes degradation by the ubiquitin-proteosome system, and NF-κB translocates into nucleus and triggers transcription of target genes[7]. Simultaneously, MAPKs are activated 'downstream' of TRAF6 by activating MAPK kinase 6 (MKK6)[8]. In the TNFR signaling pathway, ligand stimulation leads to the recruitment of adaptor proteins, including TRADD, TRAF2 and RIP1, to the receptor complex. Genetic studies have shown that TRAF2 is responsible for MAPK activation, whereas RIP1 is required for NF-κB activation[1,4].

Transforming growth factor-β–activated kinase 1 (TAK1), a member of the MAPK kinase kinase (MAPKKK) family, was originally identified as a kinase involved in TGF-β signaling[9]. TAK1 is evolutionally conserved, and drosophila TAK1 is critical for antibacterial innate immunity[10]. In addition, TAK1 functions as an 'upstream' signaling molecule of NF-κB and MAPKs in IL-1R signaling pathways. Furthermore, TAK1 is activated by TNF, bacterial lipopolysaccharide (LPS) and latent membrane protein 1 from Epstein-Barr virus[11–13]. Activated TAK1 is recruited to TRAF6 and TRAF2 complexes in response to IL-1R and TNFR stimulation, respectively. A point mutation in the gene encoding TAK1 altering its ATP-binding domain abolishes both its kinase activity and its ability to activate IKKs and MAPKs[8]. TAK1 forms a complex with its association partners, TAB1, TAB2 and TAB3 (refs. 14–17). It has been proposed that TRAF6-mediated K63-linked

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polyubiquitination is required for the activation of TAK1. Activated TAK1 complex phosphorylates IKKs and MKK6, which activate NF-κB and MAPKs, respectively. *In vitro* studies have shown that expression of TAK1 together with TAB1 enhances activation of a NF-κB reporter gene[8]. Reciprocally, 'knockdown' of *Map3k7*, the gene encoding TAK1, in HeLa cells by RNA interference results in abrogation of IL-1β- and TNF-induced NF-κB activation[11].

The function of TAB1 and TAB2 has been assessed by examination of mouse models lacking genes encoding these proteins. Analysis of TAB2-deficient mice has shown that TAB2 is dispensable for IL-1R signaling[18]. Studies of TAB1-deficient mice have shown that TAB1 is involved in TGF-β signaling[19]. However, the function of TAB1 in IL-1R signaling *in vivo* has not been described. Therefore, it is still unclear whether TAK1-binding proteins are essential for TAK1 activation or if the TAK1 complex itself is dispensable for NF-κB and MAPK signaling *in vivo*. Although the function of TAK1 in drosophila innate immune response has been studied extensively[10], its involvement in the mammalian TLR system is not well understood.

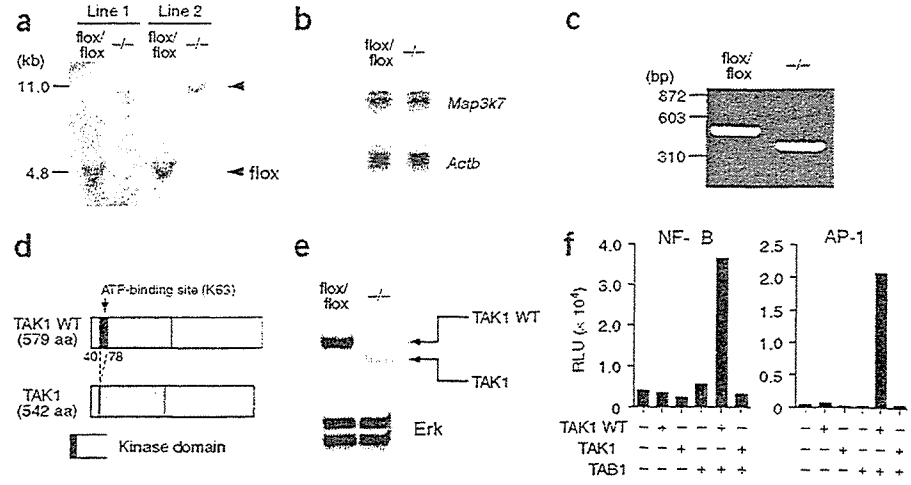

Figure 1 Establishment of *Map3k7* $^{-/-}$ MEFs. (**a**) Southern blot analysis of genomic DNA from *Map3k7* $^{-/-}$ ($^{-/-}$) and control *Map3k7*$^{flox/flox}$ (flox/flox) MEFs, after digestion with *Xba*I and *Eco*RI (probe, **Supplementary Fig. 1** online). Right and left margins, positions of 11.0-kb floxed and 4.8-kb Δ fragments. (**b**) RNA blot analysis of total RNA prepared from *Map3k7* $^{-/-}$ and *Map3k7*$^{flox/flox}$ MEFs. The 5′ ends of *Map3k7* and *Actb* (encoding β-actin) cDNA fragments were used as probes. (**c**) RT-PCR analysis of total RNA from MEFs, using primers amplifying the encoding region of exons 1–3. (**d**) Predicted structure of TAK1Δ, which lacks the ATP-binding site (arrow) required for kinase activity. WT, wild-type; aa, amino acids. (**e**) Immunoblot analysis of lysates of *Map3k7* $^{-/-}$ and *Map3k7*$^{flox/flox}$ MEFs (antibodies, right margin). (**f**) NF-κB- or AP-1-dependent reporter assay. HEK293 cells were transiently transfected with plasmids (below graphs) plus an NF-κB-dependent (left) or an AP-1-dependent (right) luciferase reporter plasmid. Then, 36 h after transfection, luciferase activity (RLU, relative light units) in whole-cell lysates was measured. Data are representative of three independent experiments.

Here we have examined the function of TAK1 *in vivo* by gene targeting using the Cre-*lox*P system. *Map3k7* deficiency in the germline resulted in early embryonic death. Therefore, we generated TAK1-deficient (*Map3k7* $^{-/-}$) mouse embryonic fibroblasts (MEFs) by *in vitro* introduction of Cre in MEFs homozygous for *lox*P-flanked (floxed) *Map3k7* alleles (*Map3k7*$^{flox/flox}$). First we examined the function of TAK1 in IL-1R and TNF signaling using TAK1-deficient MEFs and found that TAK1 was required for IL-1β- and TNF-induced NF-κB and Jnk activation as well as cytokine production. Next we analyzed TLR- and B cell receptor (BCR)–mediated signaling using B cells as a model. B cell–specific deletion of TAK1 resulted in considerably impaired B cell activation in response to various stimuli, including nonmethylated CpG DNA (a ligand for TLR9), polyinosine-polycytidylic acid (poly(I:C); a ligand for TLR3), LPS (a ligand for TLR4), CD40 and BCR crosslinking. Furthermore, LPS and CpG DNA failed to activate Jnk and NF-κB in TAK1-deficient B cells, indicating that TAK1 is essential for activating these signaling pathways. Notably, although BCR crosslinking on TAK1-deficient B cells also demonstrated defective Jnk activation, activation of NF-κB as well as expression of NF-κB target genes was comparable to that of wild-type cells. Our conditional TAK1-deficient mouse model therefore shows that TAK1 is essential for TLR, IL-1R, TNFR and BCR cellular responses and signaling pathways leading to the activation of Jnk and/or NF-κB.

RESULTS

Map3k7 $^{-/-}$ mice die early *in utero*

To investigate the function of TAK1 *in vivo*, we generated mice with conditional deletion of a *Map3k7* allele. We constructed a gene-targeting vector by placing *lox*P sites flanking exon 2 of mouse *Map3k7*, which encodes a part of the kinase domain of TAK1, including its ATP-binding site (Lys63), and a floxed neomycin-resistance gene into an intron 1 of *Map3k7* (**Supplementary Fig. 1** online). To generate mice heterozygous for deletion of this *Map3k7* allele (*Map3k7*$^{+/-}$ mice), we mated mice with one floxed allele and one wild-type allele (*Map3k7*$^{flox/+}$ mice) with a mice of a transgenic line expressing Cre in germ cells. We confirmed deletion of *Map3k7* in the germline by Southern blot analysis (**Supplementary Fig. 1** online). Of about 90 newborn pups obtained by intercrossing *Map3k7*$^{+/-}$ mice, we obtained no *Map3k7* $^{-/-}$ mice, indicating that the TAK1 deficiency is embryonically lethal (**Supplementary Fig. 1** online). Although we identified *Map3k7* $^{-/-}$ embryos on embryonic day 9.5 (E9.5) in normal mendelian ratios, we found no *Map3k7* $^{-/-}$ fetuses in decidua containing normal fetuses after E10.5 (**Supplementary Fig. 1** online).

Establishment of *Map3k7* $^{-/-}$ MEFs

As *Map3k7* $^{-/-}$ MEFs obtained from E9.5 embryos failed to grow, we prepared MEFs from *Map3k7*$^{flox/flox}$ mice. To generate TAK1-deficient MEFs, we excised the floxed genomic fragment by retroviral expression of Cre protein together with green fluorescent protein (GFP). We sorted GFP$^+$ cells by flow cytometry. Southern blot analysis showed that complete conversion of the floxed allele to the deleted (Δ) allele was achieved in GFP$^+$ cells from two lines of *Map3k7* $^{-/-}$ MEFs (**Fig. 1a**). However, we detected *Map3k7* transcripts in *Map3k7* $^{-/-}$ MEFs with same migration and intensity as that of *Map3k7*$^{flox/flox}$ MEFs (**Fig. 1b**). RT-PCR analysis using primers to amplify the region of exons 1–3 showed a product with faster migration in *Map3k7* $^{-/-}$ cells (**Fig. 1c**). Nucleotide sequence analysis of the product showed that the deletion of exon 2 from TAK1 cDNA was in-frame, indicating that Cre-mediated deletion led to the production of an altered TAK1 (TAK1Δ; **Fig. 1d**). Immunoblot analysis showed weak expression of TAK1Δ in *Map3k7* $^{-/-}$ cells (**Fig. 1e**). To confirm that TAK1Δ lacked