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Co-administration of cholera toxin and apple polyphenol extract as a novel and safe mucosal adjuvant strategy

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ABSTRACT

Although native cholera toxin (CT) is an extremely effective adjuvant, its toxicity prevents its use in humans. We report here that apple polyphenol extract (APE), obtained from unripe apples, reduces CT-induced morphological changes and cAMP accumulation. Based upon this finding, we have attempted to design a novel, effective and safe mucosal vaccine by using CT with several dosages of APE as nasal adjuvants. Mice nasally immunized with OVA plus CT and an optimal dosage of APE showed significantly reduced levels of inflammatory responses as well as total and OVA-specific IgE antibodies when compared with mice given without APE. However, levels of both mucosal and systemic OVA-specific antibody responses were maintained. Further, APE significantly down-regulated accumulation of CT in the olfactory nerves and epithelium. In summary, an optimal dosage of APE would take full advantage of mucosal adjuvant activity of native CT without any toxicity for application in humans.

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1. Introduction

The enterotoxins, native cholera toxin (CT) produced by *Vibrio cholerae*, and heat-labile toxin (LT) produced by *Escherichia coli*, serve as powerful adjuvants when co-administered with unrelated proteins via mucosal routes [1–3]. These enterotoxins enhance mucosal secretory IgA (S-IgA) and systemic IgG and IgA antibody (Ab) responses to co-administered protein antigens (Ags); however, they are unsuitable for use in humans due to their toxicity. Both CT and LT cause severe diarrhea when orally administered and central nervous system (CNS) toxicity when nasally administered [4–9]. Furthermore, safety concerns were aroused when a commercial nasal vaccine using LT as an adjuvant was withdrawn from the market due to possible association with side effects such as rhinorrhea, headache and, most seriously, facial palsy [9–12].

Four principal approaches have been developed for eliminating the toxic effects of CT while retaining its efficacy as an adjuvant; some mutants and chimeric molecules were developed to avoid the risks of CT in mucosal tissues and CNS. First, recombinant CT-B

was employed as a mucosal adjuvant since the A subunit of CT (CT-A) is responsible for toxicity [13,14]. Second, in order to establish molecules that are nontoxic but retain adjuvant activity, researchers have created nontoxic mutant CTs (mCTs) by substituting a single amino acid in the ADP-ribosyltransferase active center [3,15] and double mutant CTs (dmCTs) by mutating the ADP-ribosyltransferase active center and the COOH-terminal of CT-A2 to influence the movement of CT from the Golgi apparatus to the endoplasmic reticulum [16]. Though nasal immunization with CT-induced nerve growth factor- β (NGF- β), mCT E112K did not elicit any neuronal damage based upon NGF- β production in the CNS [17]. Further, native CT accumulated in the olfactory bulbs (OBs) whereas dmCT did not [16]. Third, CT-A1 was fused to a dimer of the Ig-binding D region of *Staphylococcus aureus* protein A (CTA1-DD) to avoid the binding of CT to all nuclear cells [18–20]. In this approach, CTA1-DD does not induce inflammatory responses and does not accumulate in the OBs [20]. Finally, CT-LT chimeric molecules were constructed that differed from CT in their biological and adjuvant activity [21,22]. Nasal administration of mCT-A/LT-B, one of the CT-LT chimeric molecules, did not enhance IgE Ab responses [23].

We assessed both the efficacy and safety of administering apple polyphenol extract (APE), a crude polyphenol extracted from unripe apples, together with native CT to mitigate its toxic effects. The

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ability of the enzymatic and biological properties of APE has already been established [24,25]. APE inhibits ADP-ribosylation *in vitro* and reduces CT-induced fluid accumulation in the intestines of mice. Moreover, it has been reported that oligomeric and polymeric catechins in APE are the major compounds inhibiting ADP-ribosylation [24]. As to the safety of APE, an acute toxicity test and a sub-chronic toxicity test in rats receiving oral APE at a dosage of 2000 mg/kg showed no significant hematological, clinical, chemical, histopathological, or urinary effects [26]. Further, in a pilot study, 10 mg/kg/day of APE proved to be nontoxic to humans when administered APE orally for 8 weeks [27].

Using recombinant CTs as mucosal adjuvants has been explored by many investigators, and these strategies have been shown to be effective mucosal adjuvants with little or no toxicity. Here, we introduce a new strategy for rendering CT a clinically suitable mucosal adjuvant. Our strategy differs radically from those outlined previously because it employs native CT, not mutant or chimeric CT. In this study, we are attempting to harness the full adjuvanticity of native CT while also guarding against toxic side effects through the use of APE.

2. Materials and methods

2.1. Toxicity and bioassay of CT *in vitro*

APE was kindly provided by Dr. Akio Yanagida (Institute for Production Research and Development, The Nikka Whisky Distilling Co., Ltd., Chiba, Japan). Apples and preparation methods of APE were described elsewhere [28]. The ability of CT (List Biological Laboratories, Campbell, CA) with or without several concentrations of APE to induce toxic effects on cultured mouse Y-1 adrenal tumor cells was assessed as described previously [29]. The cells were examined by light microscopy for typical rounding, and the concentration of CT required to initiate cell rounding (EC₁) was determined. cAMP was quantitated as described elsewhere [15]. Intracellular cAMP measurement was done with an enzyme immunoassay kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). The quantity of protein was analyzed for the same samples using a Coomassie Protein Assay Reagent (PIERCE, Rockford, IL), and the levels of cAMP were expressed as pmol of cAMP/mg of protein.

2.2. Immunization of mice

Five-week-old C57BL/6 mice (*H-2^b*) were purchased from CLEA Japan, Inc. (Tokyo, Japan). Mice were acclimated to the experimental animal facility for 1 week before being used in experiments and were maintained in the facility under specific pathogen-free conditions. All experimental procedures were carried out following the guidelines established by The Committee of Animal Experiments, Iwate Medical University. For nasal immunization, the mice were lightly anesthetized with ketamine before being immunized with a 5- μ l aliquot (2.5 μ l/nosril) of PBS containing 100 μ g of ovalbumin (OVA; Sigma–Aldrich, St. Louis, Mo) and 0.5 μ g of CT with or without specified dosages of APE, with a 5-min rest between each inoculation. Mice were immunized three times at weekly intervals in all experiments. The dosage of CT and the immunization schedule were largely the same as those used in previous studies of CT as mucosal adjuvant for mice immunized nasally [3,16,22,23,30,31].

2.3. Histopathologic analysis

We assessed the inflammatory responses in the nasal cavity of mice given nasal OVA plus CT with or without specified dosages of APE. One week after the last immunization, the head of each mouse was fixed in 10% neutral-buffered formalin and subsequently

immersed in 100% ethanol. After decalcification with 10% formic acid–10% formalin at room temperature for 3 days, the tissues at the frontal section were cut into 3-mm-thick slices and embedded in paraffin. Three- μ m-thick histological sections were stained with hematoxylin and eosin. Histopathologic evaluation of inflammatory responses in the nasal cavity was performed using light microscopy.

The severity of the lesions in each mouse was scored on a 1–4 grade based on the thickness of the inflammatory cell layer (excluding the lymph follicle) as follows: (1) less than five cells thick; (2) 5–10 cells thick; (3) inflammatory cell layer more than 10 cells thick covering less than 30% of the mucosal surface; and (4) inflammatory cell layer more than 10 cells thick covering more than 30% of the mucosal surface of the nasal cavity. Since the first two slices from the nasal apex exhibited higher inflammatory responses in every mouse, the inflammatory grading was examined in these two sections and a mean calculated for each mouse.

2.4. ELISA for inflammatory cytokines

Mice were nasally immunized once with a 5- μ l aliquot (2.5 μ l/nosril) of PBS containing 100 μ g of OVA and 0.5 μ g of CT with or without specified dosages of APE or PBS alone as a negative control. Six hours after the immunization, nasal washes were obtained by injecting 1 ml of PBS on three occasions into the posterior opening of nasopharynx with a hypodermic needle [32]. The inflammatory cytokines (IL-1 β , IL-6, and TNF- α) in the nasal wash samples were assessed by cytokine-specific ELISAs (Quantikine®; R&D Systems, Minneapolis, NE). The detection limits of the ELISA for IL-1 β , IL-6, and TNF- α were 7.8, 7.8, and 23.4 pg/ml, respectively.

2.5. Real-time quantitative PCR

The levels of inflammatory cytokine mRNA expression in the nasal tissues and the CNS of mice were determined by a real-time quantitative reverse transcription PCR. Brains, nasal septums including nasal mucosa and palates including nasopharyngeal-associated lymphoreticular tissue (NALT) were rapidly removed. Brains and OBs were isolated as previously described [33]. After isolation of the OBs, the brains were cut into 1-mm-thick slices, and the first two slices (containing tissue from the frontal region and basal ganglia, respectively) were used. Samples were immediately frozen in dry ice and stored at –80 °C until use. Total RNA of the samples was extracted and 1 μ g of total RNA was reverse transcribed [34]. The PCR protocols and the sequences of the primers of IL-1 β , IL-6, TNF- α and GAPDH have been described elsewhere [34]. To confirm amplification specificity, the PCR products from each primer pair were subsequently subjected to a dissociation curve analysis. To determine normalized cytokine mRNA values, the values for IL-1 β , IL-6, and TNF- α were divided by the endogenous reference GAPDH values of each sample. To determine the relative quantity of cytokine mRNA levels, the normalized cytokine mRNA values were divided by the normalized calibrator values. The calibrators were obtained from the unstimulated control group (PBS alone); the cytokine mRNA level in an unstimulated control was defined as 1 arbitrary unit.

2.6. Trafficking analysis of CT

The distribution of CT in the NALT, olfactory nerves and epithelium (ON/E) and OBs were examined using acridinium-labeled CT [16,35]. Mice were nasally administered 0.5 μ g of acridinium-labeled CT together with or without specified dosages of APE. After 6 h, the NALT, ON/E and OBs were isolated, and the levels of acridinium present were determined [16]. The adjuvanticity of CT was not affected by acridinium labeling. Thus, when mice were nasally

immunized with OVA plus CT or acridinium-labeled CT, comparable levels of OVA-specific Ab responses were noted (data not shown).

2.7. Determination of IgE Abs

Total IgE Abs titers in plasma were determined using a sandwich ELISA [23]. Plasma samples were collected 1 week after the last immunization. Concentrations of total IgE Abs in plasma were calculated using a standard curve. OVA-specific IgE Abs responses were determined by an IgE capture method [15,16]. TMB+ (3,3',5,5'-tetramethylbenzidine; DAKO, Carpinteria, CA) were used as substrate-chromogen. Endpoint titers were expressed as the reciprocal log₂ of the last dilution that gave an optical density at 450 nm (OD₄₅₀) of ≥0.1 OD unit above the OD₄₅₀ of negative controls after a 15-min incubation. Incubations were terminated by addition of 0.5 M H₂SO₄.

2.8. Detection of OVA or CT-B-specific Abs

One week after the last immunization, blood and mucosal secretions (fecal extracts, saliva, nasal washes, and vaginal washes) were collected. The collecting methods used have been previously described elsewhere [32,36,37]. Titers of OVA-specific or CT-B-specific Ab in samples were determined by an endpoint ELISA [36]. For each experiment, vaginal washes were pooled from four mice. Endpoint titers were expressed as the reciprocal log₂ of the last dilution that gave an OD at 450 nm (OD₄₅₀) of ≥0.1 OD unit above the OD₄₅₀ of negative controls after a 15-min incubation.

2.9. Detection of OVA-specific Ab-forming cells (AFCs)

Single-cell suspensions were obtained from NALT, nasal lamina propria (n-LP), small intestinal lamina propria (i-LP), mesenteric lymph nodes (MLNs), submandibular lymph nodes (SMLNs), submandibular glands (SMGs), spleen and axially lymph nodes (ALNs) were prepared as described elsewhere [30,36,37]. OVA-specific IgG and IgA AFCs in the mucosal and systemic tissues of mice were determined by ELISPOT assay [3,23]. The numbers of OVA-specific AFCs were quantitated with the aid of a stereomicroscope.

2.10. GM1-binding assay

Nunc immunoplates were coated with a ganglioside GM1. Specified concentrations of APE and CT (final concentration: 10 ng/ml) were mixed well and added into each well after blocking with 1% BSA. To detect GM1 binding by CT, we used mouse anti-CT serum at a dilution of 1:1000, followed by horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) Abs. The plates were developed at room temperature with TMB+ substrate-chromogen. Incubations were terminated by addition of 0.5 M H₂SO₄, and optical density was measured at 450 nm.

2.11. Statistics

The results are expressed as the mean ± the standard error of mean (SEM). Normally distributed variables were compared by the two-tailed Student's *t* test and nonnormally distributed variables by the two-tailed Mann–Whitney *U* test. A *p* value of <0.05 was considered significant.

3. Results

3.1. APE reduced toxicity of CT in vitro

If the toxicity of CT could be mitigated or eliminated by the use of APE, its efficacy as a mucosal adjuvant could be fully harnessed

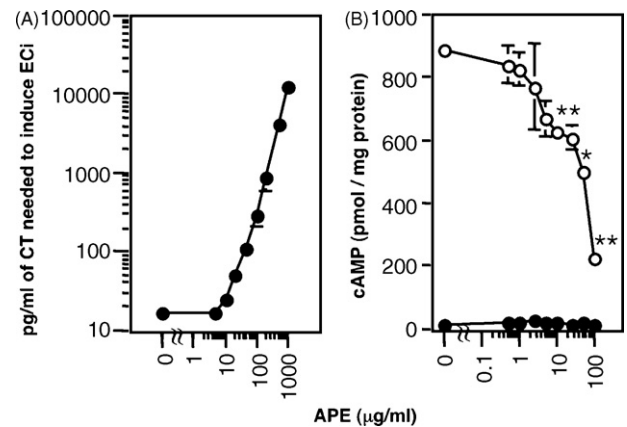


Fig. 1. Morphological responses of Y-1 cells and cAMP induction in CHO cells by CT with or without APE. (A) Morphological changes of Y-1 cells were assessed by culture with CT and several concentrations of APE. The data show the concentration of CT initiating rounding of Y-1 cells as ECi. The data for ECi are shown as the mean concentration ± one SEM for three different experiments. (B) CHO cells were cultured with specified concentrations of APE alone (closed circle) or together with 100 ng/ml of CT (open circle). The levels of cAMP were expressed as pmol of cAMP/mg of protein. The data for cAMP induction are shown as the mean level ± one SEM for three different experiments. Significant differences between the CT-only group and the CT plus APE group are indicated by asterisks (**p* < 0.05, ***p* < 0.005).

in humans. To assess the ability of APE to reduce CT toxicity, we initially performed a Y-1 adrenal cell assay and determining ECi. When CT was added into the Y-1 cell cultures together with varying concentrations of APE, the toxicity of CT was reduced in a dose-dependent manner (Fig. 1A). At a molar rate of 1:1000 and 1:100 of CT and APE, a combination of CT/APE had 1/731 and 1/17 fewer toxic activities for ECi, respectively, than did CT alone. Since the morphological response of cells to CT has been shown to be dependent upon adenylylase-mediated increases in cAMP [38], we next assessed the induction of cAMP by CT with or without APE. When more than 10 μg/ml (1:100 to 1:1000) of APE was administered, cAMP levels were significantly decreased (Fig. 1B). APE alone did not change cAMP levels in controls. These results demonstrate that APE reduced the ADP-ribosyltransferase activity of CT *in vitro*.

3.2. APE reduced inflammatory responses induced by CT in vivo

Histopathologic studies allowed us to compare the inflammatory responses in the nasal cavity of mice given nasal OVA plus CT with one of several specified dosages of APE and without APE. The severity of the inflammatory response was scored as described in Section 2, and representative histopathology images of each grade are shown in Fig. 2A. In all four mice nasally immunized with OVA and 0.5 μg of CT without APE, grade 3 or 4 inflammatory responses (with an inflammatory layer ≥10 cells thick) were observed (Fig. 2B). However, inflammatory responses were reduced in mice given nasal OVA plus 0.5 μg of CT in the presence of 50–500 μg (1:100 to 1:1000) of APE. Indeed, grade 1 inflammatory responses (inflammatory layer <5 cells thick) were observed in two of the four mice given 500 μg (1:1000) of APE (Fig. 2B).

Since it has been shown that nasal CT redirected co-administrating tetanus toxoid into the ON/E and NALT within 3 h, with highest levels of IL-1β and IL-6 expression over the next 3 h [39], we next assessed IL-1β, IL-6, and TNF-α productions in nasal washes after 6 h of administration of vaccines in order to detect early inflammatory responses. The mean level of IL-6 in nasal washes of mice nasally immunized with OVA plus CT without APE was 601.9 ± 35.7 pg/ml. As a reference, the level of IL-6 in nasal washes of mice administered PBS alone was 13.9 ± 4.6 pg/ml. The

IL-6 levels in nasal washes of mice given nasal OVA and 0.5 μg of CT together with 100–500 μg (1:200 to 1:1000) of APE were significantly lower than in mice immunized with OVA plus CT. Tenfold lower levels of IL-6 were observed in mice immunized with OVA and CT together with 500 μg of APE when compared with mice nasally immunized with OVA and CT alone (Fig. 2C). In contrast, essentially no IL-1 β or TNF- α was detected in nasal washes (data not shown).

Furthermore, real-time RT-PCR was performed to quantify levels of inflammatory cytokine-specific mRNA expression in nasal tissues and brains. The levels of IL-1 β -, IL-6-, and TNF- α -specific mRNA were significantly higher in the palates, including the NALT and nasal septums with nasal mucosa of mice administrated OVA plus CT than negative control mice. Though IL-1 β and TNF- α were not detected in nasal wash samples by ELISA, levels of IL-1 β -, IL-6-, and TNF- α -specific mRNA expression in nasal tissues of mice administrated OVA plus CT with 100 or 500 μg of APE were significantly decreased when compared with mice given CT without APE. In particular, the palates and nasal septums of mice with 500 μg of APE exhibited significantly low level of IL-6-specific mRNA expression (a decrease of 83%, $p = 0.0003$, and 94%, $p = 0.002$, respectively) (Fig. 2D). In contrast, no significant changes in inflammatory cytokine-specific mRNA levels in OBs, frontal regions and basal ganglia regions were noted (data not shown).

3.3. APE reduced accumulation of CT in the nasal and CNS tissues

Our studies showed that APE reduced inflammatory responses and inflammatory cytokines production by CT in the nasal tissues. Though the biological and pathogenic consequences of CT in the CNS is not understood, it is most important to confirm CNS toxicity for the safety of this nasal vaccine. CT accumulation levels were significantly lower in NALT and ON/E of mice nasally immunized

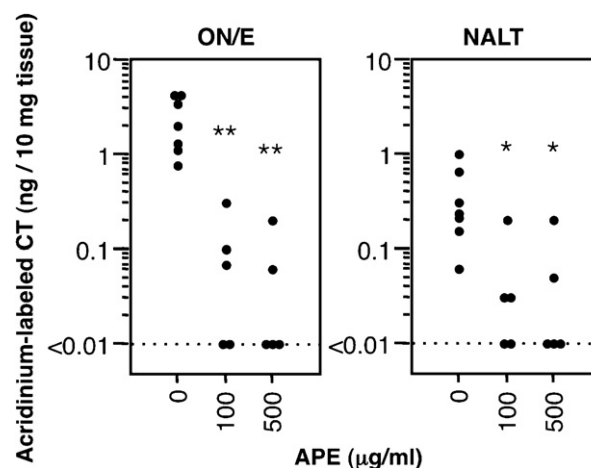


Fig. 3. Comparison of the distribution of CT in olfactory tissues of mice immunized with OVA plus CT with or without nasal APE. Distribution of acridinium-labeled CT into the NALT and ON/E was determined 6 h after nasal administration of 0.5 μg of CT with 0, 100 or 500 μg of APE. The NALT and ON/E were collected and analyzed for presence of acridinium-labeled CT. Data are expressed as ng of CT/10 mg weight of tissue. The CNS tissues of mice given nasal PBS were examined for background levels of luminescent. These control values were subtracted from each experimental value. Significant differences between the CT-only group and the CT plus APE group are indicated by asterisks (* $p < 0.05$, ** $p < 0.005$).

with OVA plus 0.5 μg of acridinium-labeled CT together with 100 and 500 μg (1:200 and 1:1000) of APE than in mice immunized with OVA plus CT after 6 h (Fig. 3). In contrast, the acridinium-labeled CT in OBs was very low or undetectable (<0.02 ng/10 mg tissue) and there were no significant changes in groups immunized with or without APE (data not shown). These results clearly show that CT

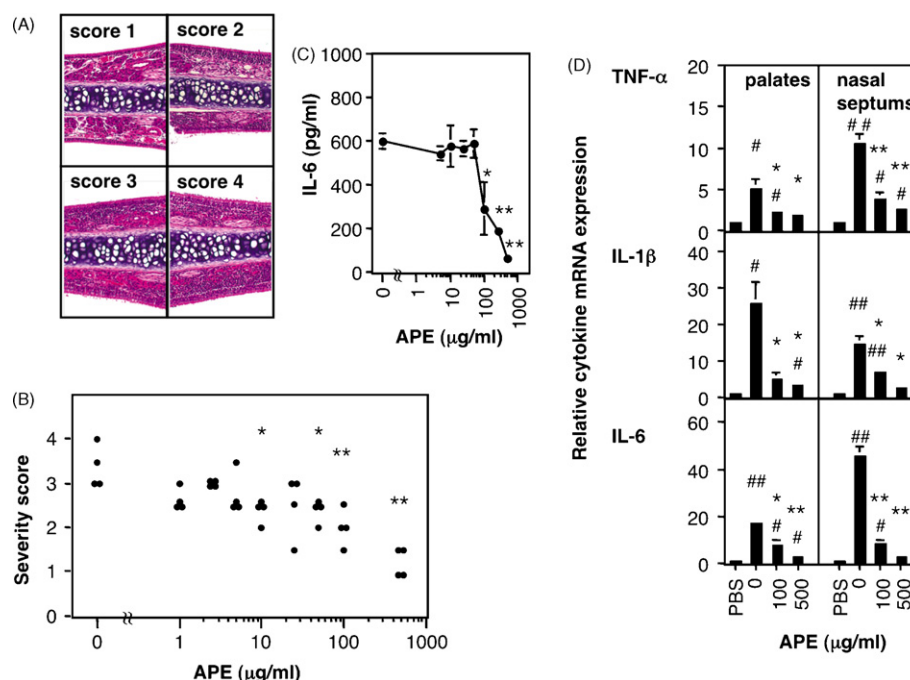


Fig. 2. Inflammatory responses in the nasal cavity of mice nasally immunized with OVA plus CT with or without APE. (A) Frontal cross-sections of the nasal cavity of mice were taken at 1 week after the last immunization. The severity of the lesions in each mouse was scored on a 1–4 grade based on the thickness of the inflammatory cell layer. Representative histopathology images of each grade are shown. (B) Inflammation was graded in the first two slices from the nasal apex of each mouse, and a mean calculated to determine the inflammatory score for each mouse. (C) The concentrations of IL-6 in the nasal washes of mice immunized with OVA plus CT with or without APE were determined by IL-6-specific ELISA. The data are shown as the mean level $\pm$ one SEM for 12 mice in each experimental group, and the data are representative of three separate experiments. (D) Real-time quantitative RT-PCR analysis was performed to compare cytokine-specific mRNA expression in the nasal tissues. Results are presented as fold decrease or increase compared with mice administered PBS alone. The data are shown as the mean level $\pm$ one SEM for 4 mice in each experimental group and the data are representative of two separate experiments. Significant differences between the CT-only group and CT plus APE group are indicated by asterisks (* $p < 0.05$, ** $p < 0.005$), and the PBS alone group and CT plus APE group are indicated by squares (# $p < 0.05$, ## $p < 0.005$).

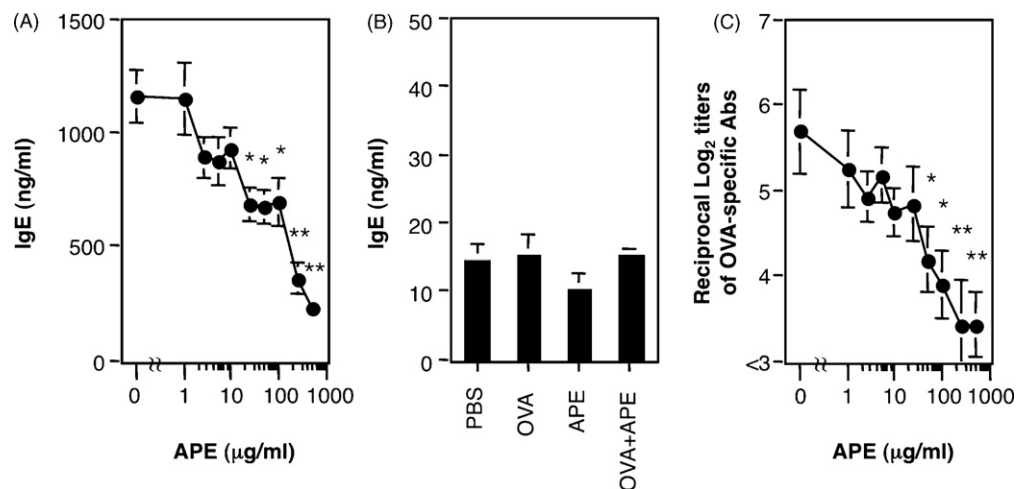


Fig. 4. Total and OVA-specific IgE Ab responses in plasma of mice given nasal OVA plus CT with or without APE. Mice were nasally immunized with OVA plus CT with different doses of APE three times weekly intervals. Plasma samples were collected 1 week after the last nasal immunization. (A) The levels of total IgE Abs were determined by sandwich ELISA. (B) As reference data, the levels of total IgE Abs in the plasma of mice administered PBS alone, 100 μg of OVA, 500 μg of APE, or 100 μg of OVA plus 500 μg of APE were determined. (C) The titers of OVA-specific IgE Abs in the plasma of mice co-administered different doses of APE were determined by OVA-specific IgE ELISA. The data are shown as the mean concentration $\pm$ one SEM for 12 mice in each experimental group, and the data are representative of three separate experiments. Significant differences between the CT-only group and the CT plus APE group are indicated by asterisks (* p < 0.05, ** p < 0.005).

fails to accumulate in the ON/E in the presence of the optimal dose of APE.

3.4. APE reduced total and OVA-specific IgE Ab responses

In addition to its toxicity, one of the drawbacks of CT is the induction of high IgE Ab responses to bystander Ags. To determine whether the combination of APE and CT down-regulates IgE Ab responses, we assessed total IgE Abs in the plasma of mice nasally immunized with OVA plus CT with or without APE. Significantly lower level of total IgE Ab responses were seen in the plasma of mice given 25–500 μg (1:50 to 1:1000) of nasal APE than in those without APE (Fig. 4A). The control groups of mice given nasal PBS, OVA alone, 500 μg of APE, or OVA plus 500 μg of APE, resulted in identical low total IgE Ab titers (Fig. 4B). We next assessed OVA-Specific IgE Abs in the plasma of mice nasally immunized with OVA plus CT with or without APE by endpoint ELISA. Significant reductions in OVA-specific IgE Ab titers were observed in the plasma of

mice given nasal OVA plus 0.5 μg of CT together with 50–500 μg (1:100 to 1:1000) of APE (Fig. 4C).

3.5. APE maintained induction of OVA-specific IgA Abs in mucosal secretions

It is important to show co-administration of APE does not affect the adjuvanticity of nasal CT. In this regard, mice were nasally immunized with OVA plus CT alone or together with one of several specified dosages of APE. One week after the last immunization the levels of OVA-specific IgA Abs in mucosal secretions (fecal extracts, nasal washes, saliva and vaginal washes) were examined by endpoint ELISA. Mice nasally immunized with OVA plus 0.5 μg of CT together with 1–250 μg (1:2 to 1:500) of APE showed OVA-specific IgA Abs in mucosal secretions that were essentially identical to those observed in mice immunized with OVA plus CT alone (Fig. 5). In contrast, mice given nasal OVA plus 0.5 μg of CT together with 500 μg (1:1000) of APE revealed significantly reduced titers of OVA-

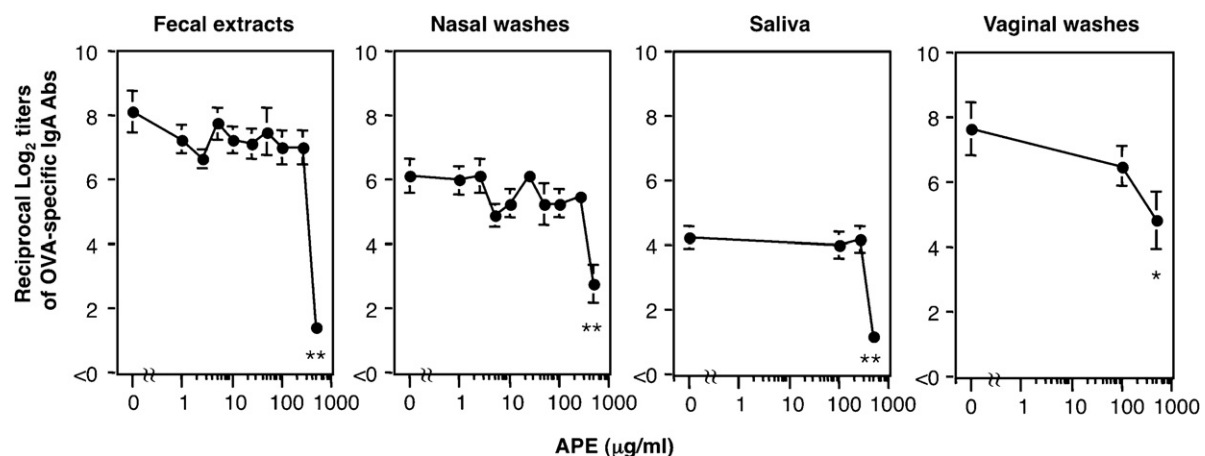


Fig. 5. OVA-specific IgA Ab responses in mucosal secretions of mice nasally immunized with OVA plus CT with or without nasal APE. Mice were nasally immunized with OVA plus CT with different doses of APE three times weekly intervals. Mucosal secretions (fecal extracts, nasal washes, saliva, and vaginal washes) were collected 1 week after the last immunization. OVA-specific IgA Ab levels in the mucosal secretions were determined by OVA-specific endpoint ELISA. The data for fecal extracts, nasal washes, and saliva are shown as the mean titer $\pm$ one SEM for 12 mice in each experimental group, and the data are representative of three separate experiments. The samples of vaginal washes were pooled from four mice, and six pooled samples were assessed by ELISA. Significant differences between the CT-only group and CT plus APE group are indicated by asterisks (* p < 0.05, ** p < 0.005).

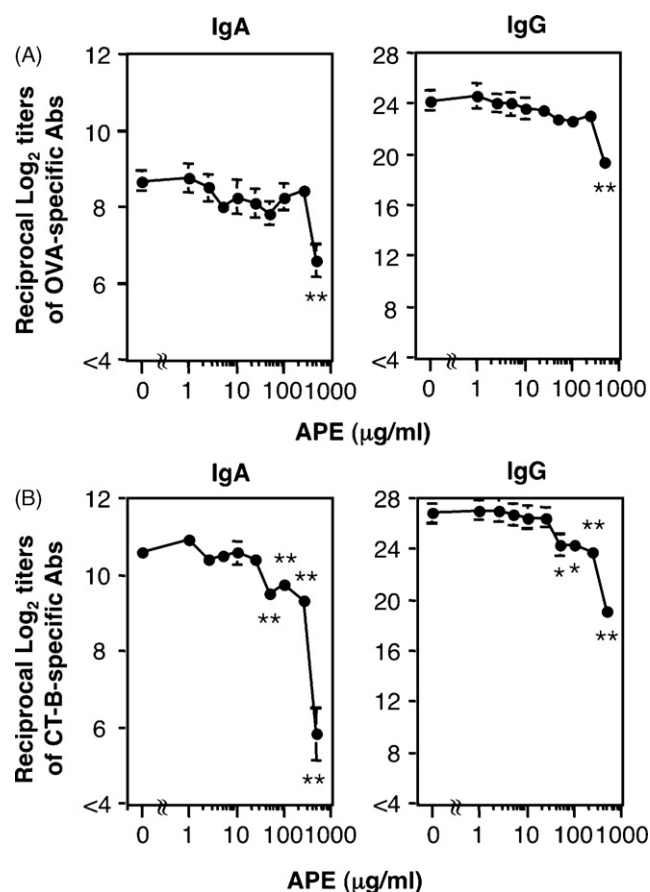


Fig. 6. OVA- or CT-B-specific IgA and IgG Ab responses in the plasma of mice immunized with OVA plus CT with or without nasal APE. Mice were immunized as described in Fig. 5 legend and plasma samples of each group of mice were collected 1 week after the last nasal immunization. The titers of OVA- (A) or CT-B- (B) specific IgA and IgG Abs in the plasma were determined by OVA- or CT-B-specific endpoint ELISA. The data are shown as the mean titer $\pm$ one SEM for 12 mice in each experimental group and the data are representative of three separate experiments. Significant differences between the CT-only group and the CT plus APE group are indicated by asterisks (* p < 0.05, ** p < 0.005).

specific IgA Abs in mucosal secretions (Fig. 5). These results suggest that APE dosages of less than 500 μ g (1:1000) could maintain the mucosal adjuvant ability of CT to induce Ag-specific IgA Abs in mucosal secretions.

3.6. APE maintained OVA-specific IgA and IgG Abs in plasma

We next analyzed systemic immune responses. The plasma of mice nasally immunized with OVA plus CT alone or with one of several specified dosages of APE were subjected to endpoint ELISA in order to assess the levels of OVA-specific IgA and IgG Abs. Similar levels of OVA-specific IgA and IgG Ab responses were detected in plasma of mice given 0–250 μ g (up to 1:500) of APE (Fig. 6A); however, these OVA-specific Ab titers were significantly reduced when 500 μ g (1:1000) of APE was employed. Identically, the dose-dependent APE effect was also noted for OVA-specific IgA Ab responses in mucosal secretions (Fig. 5). When CT-B-specific IgA and IgG Ab responses were examined, significantly reduced titers were observed in the plasma of mice nasally immunized with OVA plus 0.5 μ g of CT together with 50–500 μ g (1:100 to 1:1000) of APE. These results suggest that lower doses of APE could alter antigenicity of CT when compared with APE doses which influence CT adjuvanticity (Fig. 6B).

3.7. Comparison of OVA-specific Abs subclass IgG in plasma

We next investigate OVA-specific IgG subclass Ab responses in plasma of mice nasally immunized with OVA plus CT with or without APE. In all groups of mice, OVA-specific IgG1 Ab levels were highest, followed by Ab subclasses of IgG2b, IgG2a, and IgG3 (Fig. 7). As we anticipated, 500 μ g (1:1000) of APE significantly reduced OVA-specific IgG1, IgG2a, IgG2b and IgG3 Ab titers in the plasma; however, co-administration of 1–250 μ g (1:2 to 1:500) of APE showed intact IgG subclass responses (Fig. 7). When we calculated the ratio of IgG1 to IgG2a titers for each mouse, the mean values of the ratio were essentially unchanged regardless APE dosage (data not shown).

3.8. Assessment of OVA-specific AFCs in the mucosal and systemic tissues

In order to confirm OVA-specific Ab responses at the cellular level, we next compared the number of OVA-specific AFCs in the mucosal (NALT, n-LP, i-LP, MLNs, SMLNs and SMGs) and systemic (spleen and ALNs) lymphoid tissues 1 week after last nasal immunization with OVA plus 0.5 μ g of CT with 0 μ g (1:0), 100 μ g (1:200), or 500 μ g (1:1000) of APE. Significantly reduced number of OVA-specific IgA and IgG AFCs were seen in the n-LP, i-LP, SMLNs, SMGs and spleen of mice co-administered of 500 μ g (1:1000) of APE (Fig. 8).

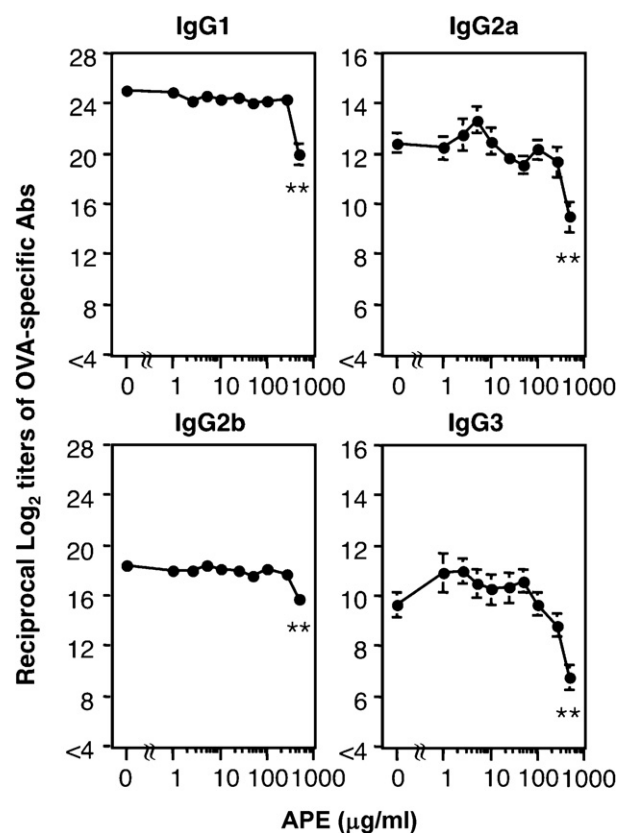


Fig. 7. OVA-specific IgG subclass Ab responses in the plasma of mice nasally immunized with OVA plus CT with or without nasal APE. Mice were immunized as described in Fig. 5 legend and plasma samples of each group of mice were collected 1 week after the last nasal immunization. The titers of OVA-specific IgG1, IgG2a, IgG2b, and IgG3 Abs in the plasma were determined by OVA-specific endpoint ELISA. The data are shown as the mean titer $\pm$ one SEM for 12 mice in each experimental group and the data are representative of three separate experiments. Significant differences between the CT-only group and the CT plus APE group are indicated by asterisks (** p < 0.005).

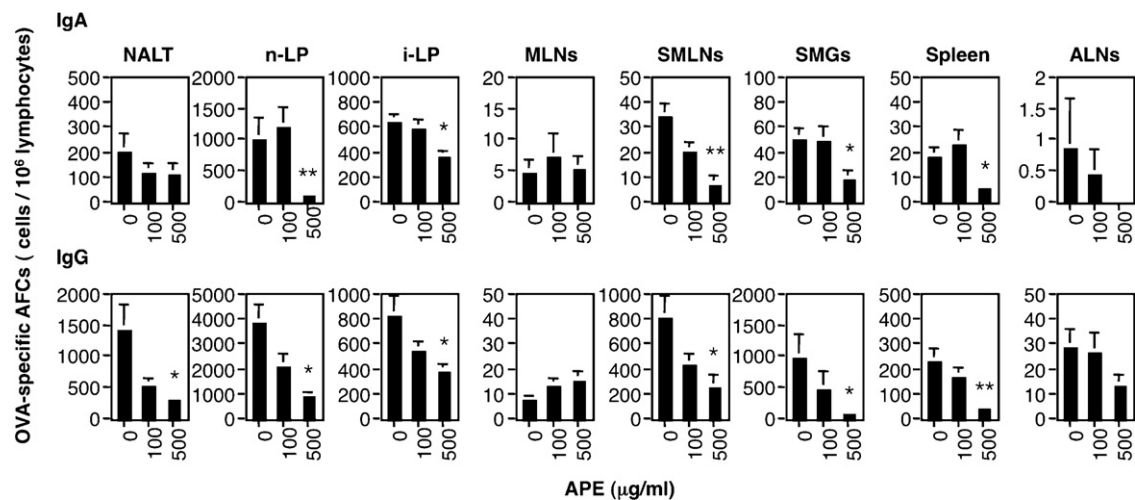


Fig. 8. Analysis of OVA-specific IgA and IgG AFCs in the mucosal and systemic tissues of mice nasally immunized with OVA plus CT with or without APE. The mice were nasally immunized with OVA plus 0.5 µg of CT alone, with 100 µg (1:200) of APE or with 500 µg (1:1000) of APE. Seven days after the last immunization, mononuclear cells isolated from NALT, n-LP, i-LP, MLNs, SMLNs, SMGs, spleen, and ALNs were determined using an OVA-specific ELISPOT assay. The data are shown as the mean number of AFCs/10⁶ cells ± one SEM for 12 mice in each experimental group and the data are representative of three separate experiments. Significant differences between the CT-only group and the CT plus APE group are indicated by asterisks (**p* < 0.05, ***p* < 0.005).

3.9. GM1 binding responses of APE complexes with CT

When a 1000-fold dosage of APE was nasally administered with CT, CT showed significant alteration of its biological activities including mucosal adjuvanticity. In this regard, we next determined whether APE influences the binding ability of CT to ganglioside GM1 using an *in vitro* binding assay. Our results showed that significant reduction of the GM1 binding ability of CT was initially detected when 10 µg (1:1000) of APE was co-cultured with CT (Fig. 9; *p* < 0.05). When higher doses (50–10,000 µg; 1:5000 to

1:1,000,000) of APE were employed, the GM1 binding ability of CT was drastically decreased (*p* < 0.005) (Fig. 9).

4. Discussion

This study provides direct evidence that co-administration of an optimal dose of APE dramatically mitigates CT toxicity without altering mucosal adjuvant activity of native CT to induce Ag-specific humoral immunity in the mucosal and systemic compartments of the mouse model. Our findings showed that the biological effects of APE on adjuvanticity and toxicity of CT were dose-dependent. At the optimal dosages of APE (molar ratio between 1:100 and 1:500 of CT and APE), CT toxicities including inflammatory responses, CNS accumulation, and IgE Ab responses were significantly reduced. Of importance, levels of OVA-specific Abs and OVA-specific AFCs were maintained with an optimal dose of APE. Further, CT binding to GM1 was essentially unchanged (Table 1). In contrast, when low dosages of APE (molar ratio between 1:2 and 1:50 of CT and APE of the combination) were used, toxicities and adjuvanticity of CT were remained intact were (Table 1). When high dosages of APE (molar ratio 1:1000 of CT and APE) were co-administered, CT lost its biological activities including toxicities and adjuvanticity (Table 1). Taken together, these findings show that a dose of APE regulates the optimal efficacy and safety of an adjuvant constituted of CT.

It has been shown that apple condensed tannin (ACT), which is an oligomeric procyanidin containing the dimer to pentadecamer of epicatechin as a unit [40], binds to both CT-A and CT-B [25]. When an optimal dose of APE was mixed with CT, CT-APE complexes were internalized to GM1-expressed cells. However, the CT-APE complex induced significantly lower cAMP levels than CT alone. These results suggest that an optimal dose of APE binds to CT-A but not CT-B subunit. In this regard, it is possible that CT, when administered with a high dose of APE, loses some of its adjuvant effect since it cannot be internalized in GM1-expressed cells. Indeed, our results showed that a high dose of APE significantly reduced CT binding to GM1. Taken together, these results suggest that APE binds first to CT-A as the primary reaction and then binds to CT-B.

It seems that the adjuvant effects achieved by a combination of CT and an optimal dosage of APE result in part from the binding of APE to CT; however, the precise mechanisms of APE in maintaining the adjuvanticity and reducing the inflammatory responses of CT remain to be elucidated. It was reported that adjuvant activity

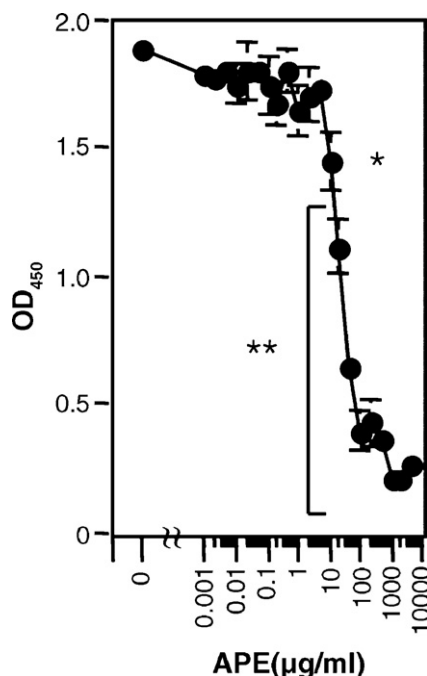


Fig. 9. Analysis of GM1 binding by CT with or without APE. A GM1 coated wells were incubated with 10 ng/ml of CT and different concentration of APE. Mouse anti-CT serum followed by horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) Abs were used to detect CT. The data are shown as the mean OD₄₅₀ ± one SEM for five different experiments. Significant differences between the CT-only group and the CT plus APE group are indicated by asterisks (**p* < 0.05, ***p* < 0.005).

Table 1
Biological characteristics and potential toxicity of CT/APE mucosal adjuvant.

CT:APE (wt:wt)		Control	Low dosage			Optimal dosage			High dosage
		1:0	1:2	1:5	1:50	1:100	1:200	1:500	1:1000
Toxicity	cAMP	–	–	–	–	*a	*	*	**a
Adverse reaction	Inflammation	–	–	–	–	*	**	NT ^b	**
	IL-6	–	–	–	–	–	*	**	**
	Total IgE	–	–	–	*	*	*	**	**
	OVA-specific IgE	–	–	–	–	*	*	**	**
CT accumulation	NALT	–	NT	NT	NT	NT	*	NT	*
	ON/E	–	NT	NT	NT	NT	**	NT	**
GM1 binding		–	–	–	–	–	–	–	*
Systemic immunity	OVA-specific								
	IgA	–	–	–	–	–	–	–	**
	IgG	–	–	–	–	–	–	–	*
	IgG1	–	–	–	–	–	–	–	**
	IgG2a	–	–	–	–	–	–	–	**
	IgG2b	–	–	–	–	–	–	–	**
	IgG3	–	–	–	–	–	–	–	**
CT-B-specific	IgA	–	–	–	–	**	**	**	**
	IgG	–	–	–	–	*	*	**	**
Mucosal immunity	OVA-specific IgA								
	Fecal extract	–	–	–	–	–	–	–	**
	Nasal wash	–	–	–	–	–	–	–	**
	Saliva	–	NT	NT	NT	NT	–	–	**
	Vaginal wash	–	NT	NT	NT	NT	–	NT	*

^a Significant differences between the CT-only group and the CT plus APE group are indicated by asterisks (* $p < 0.05$, ** $p < 0.005$).

^b Not tested.

of CT is closely related to NF- κ B translocation-mediated dendritic cell maturation [41], as well as protein kinase C (PKC)-induced pro-inflammatory cytokine production, such as IL-1 β and IL-6 [42]. Apple contains many phytochemicals such as procyanidins, phenol carboxylic acids, catechins, and flavonoids, which possess physiologic and biological functions [28,43–47]. Indeed, apple polyphenol inhibits TNF- α -induced NF- κ B activation by inhibiting the proteasomal activities instead of I κ B kinase activation [48]. Apple flavonoid also decreases TNF- α -stimulated NF- κ B signaling [49]. Further, a polyphenol-rich apple juice extract and phloridzin, the major phenolic glucoside in apple [50], inhibit PKC activity [51,52]. In addition to the above, many plant polyphenols inhibit the activity of eukaryote protein kinase [53,54] and the natural ligand that binds to its receptor [55]. These evidences indicate potential molecular mechanisms of APE for the reduction of CT-induced inflammatory responses.

It should be emphasized that polarized Th2-type T cell responses are often associated with an adverse reaction, the induction of IgE Ab and anaphylactic shock [56–58]. CT [59], mCT [3,15,31], dmCT [16], or mCT-A/LT-B [23] adjuvants are known to induce co-administered Ag-specific Th2-type T cell responses although the mutant and chimeric CTs can reduce adjuvant-induced IgE Ab titers and inflammation [15,16,23]. Our current study and others showed that APE itself did not reduce spontaneous IgE Ab levels in mice and humans [27]. However, an optimal dosage of APE, administered with OVA plus CT, diminished both total and OVA-specific IgE Ab titers in plasma without compromising the ability of CT to induce OVA-specific mucosal and systemic humoral immune responses in mice. APE binds to the high-affinity receptor for IgE Ab (Fc ϵ RI) and disturbs binding of IgE Ab to Fc ϵ RI [60]. Apple polyphenols have also been reported to suppress auricular swelling in allergic mice [61], and alleviate skin inflammation in atopic patients [27]. Though an optimal dosage of APE did not completely inhibit the production of CT-induced IgE Abs as compared with mice given PBS alone, it could help to reduce adverse reactions such as anaphylactic shock caused by CT-induced IgE Abs.

Native CT and LT pose the risk of serious adverse reactions such as inflammation and physiological epithelial cell barrier disruption in the nasal mucosa. These reactions increase the risk of toxic side effects, including the accumulation of GM1-binding adjuvants and co-administered unrelated proteins into the OBs and brain following nasal immunization [33,62,63]. In the current study, APE greatly reduced CT-induced inflammatory responses in the nasal tissues. In addition to inflammatory responses, accumulation of CT in CNS is a very critical issue in nasal vaccine development using CT as a mucosal adjuvant. Thus, it has been shown that nasal CT accumulated in the CNS tissues and induced toxicity. Indeed, a human vaccine containing inactivated influenza and native LT as an adjuvant resulted in a very high incidence of Bell's palsy [12]. In this regard, dmCTs were developed and showed their safety [16]. Although dmCTs accumulated in the ON/E for a short period, dmCTs were not transferred into the OBs [16]. Furthermore, the dmCTs that accumulated in the ON/E cleared after 24 h and were not seen in the OBs 7 days after the last immunization even though they were given three times in three consecutive administrations at weekly intervals [16]. Similarly, APE significantly reduced levels of CT accumulation in the ON/E of mice administered CT plus optimal dose of APE nasally although the transfer mechanism of CT-APE complex into the olfactory tissues could differ from that of dmCT. Since the optimal doses of APE did not significantly alter CT binding to GM1, it is possible that nasally co-administered APE prohibits CT migration into the OBs and other CNS tissues.

An ideal effective mucosal adjuvant should induce robust Ag-specific mucosal S-IgA and plasma IgG Ab responses without causing adverse reactions (e.g., IgE Ab production and inflammatory responses). The co-administration of APE together with native CT shows promise as an effective mucosal adjuvant; however, it is essential to perform additional studies in order to determine its stability and its efficacy against pathogens. Though further study is necessary, the concept of a combined native CT and APE nasal adjuvant opens up important new avenues of research for the development of a novel mucosal vaccine strategy.

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