



A CpG-containing oligodeoxynucleotide as an efficient adjuvant counterbalancing the Th1/Th2 immune response in diphtheria–tetanus–pertussis vaccine

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Abstract

Adjuvants in vaccines are immune stimulants that play an important role in the induction of effective and appropriate immune responses to vaccine component(s). Diphtheria–tetanus–pertussis (DPT) vaccine contains not only aluminum hydrate (alum) to enhance the immune response to the vaccine ingredients, but also, both for that purpose and as a principal ingredient, pertussis toxin (PT). However, both adjuvants strongly promote T helper (Th) 2 type immune responses. Th1 and Th2 type immune responses are counterbalanced in vivo, and a Th2-prone immune response is not effective against intracellular infections but promotes IgE production, which is related to allergic disease. In this study, we used the CpG motif contained in oligodeoxynucleotide (CpG-ODN), which has an adjuvant effect and also induces the Th1 response, as an adjuvant to this vaccine, and we investigated its adjuvanticity and its potential to modulate immune responses to DPT vaccine. Administration of DPT vaccine with CpG-ODN (DPT-alum/ODN) to mice significantly reduced the total IgE levels and increased the anti-PT specific IgG2a titer in serum, in comparison with ordinary DPT vaccine (DPT-alum). Moreover, we investigated the antibody response to orally administered ovalbumin (OVA) after vaccine administration. In the DPT-alum/ODN-administered group, the OVA specific IgE production in serum greatly decreased in comparison with that in the DPT-alum-administered group. These data indicate that CpG-ODN was not useful only as an efficient vaccine adjuvant but also shifted the immune responses substantially toward Th1 and modulated the Th1/Th2 immune response in DPT vaccine. These data suggested new applications of CpG-ODN as adjuvants in DPT vaccine.

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1. Introduction

Vaccine administration is a useful method of protecting children from diseases caused by pathogens to which they are susceptible in early life. Some vaccines contain adjuvant(s). Many chemicals and natural materials are known to be adjuvants and are used to enhance desired immune responses in vivo [1]. For safety reasons, great prudence is required in the

administration of adjuvants, especially those used in vaccines for human use, to young children.

Diphtheria–tetanus–pertussis (DPT) vaccine, which is given to young children, was administered to infants in order to protect them from these diseases. DPT vaccine contains two types of adjuvant, aluminum hydrate (alum) and pertussis toxin (PT). Alum has been the sole adjuvant used on a large scale for childhood vaccines for many decades. PT is a major virulence factor of *Bordetella pertussis*, the pathogen of whooping cough, and also activates the immune response and works as an adjuvant in DPT vaccine. The immune responses to alum and PT have been demonstrated

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to influence the differentiation of T lymphocyte responses toward the T helper (Th) 2 phenotype in both mice and humans [2,3]. In an animal model, PT administered with alum to rats greatly enhanced the production of IgE antibodies to unrelated antigens, even when administered 6 weeks beforehand [4]. Th2-biased immune responses enhance IgE production and play a role in causing allergic diseases [5,6], and immunoglobulin (Ig) E antibody has been demonstrated to be related to some allergic reactions in animal models [7] and in human subjects [8]. In allergic animal models, anti IgE treatment ameliorates their symptoms [9], and recently, treatment with anti human IgE monoclonal antibodies has been shown to be effective against allergic diseases [10].

Recently, bacteria-derived specific sequence patterns (CpG motif: oligonucleotide containing unmethylated cytosine and guanine dinucleotides in specific base contexts) have been shown to activate innate immunity and induce Th1 type immune responses in vivo [11]. At the cellular level, CpG activates and induces dendritic cells (DC), monocytes and macrophages into maturation [12–15], and stimulates B cell proliferation, immunoglobulin synthesis, histocompatibility complex class II expression promotion, and synthesis of Th1 cytokine (interleukin-12, IL-12; interferon-gamma, IFN- γ) induction and up-regulation of surface markers and IgM in B cells [16,17], and one study group reported that CpG induced T-bet (Th1 gene regulator) expression and inhibited IgG1 and IgE switching in B cells [18]. The use of CpG-containing oligodeoxynucleotides (ODN) for vaccine adjuvants has been studied and shown to be excellent in inducing protective immunity against parasite infections, bacterial infections and viral infections when compared with most established adjuvants in many animal models [19–22] and even in young mice [23].

Allergic disorders such as asthma, food allergies, and atopic dermatitis are increasing in children in developed countries [24,25]. Most children with allergic diseases show higher IgE titers in serum than normal children. Some studies indicate that many triggers, such as genes, environmental factors and infections, were involved in a complicated manner in inducing and exaggerating allergic diseases [26]. In this study, we investigate the effect of the adjuvants in DPT vaccine on Th1/Th2 immune responses and the antibody responses after vaccine administration in a young mouse model. Next, we added CpG-ODN to the DPT vaccine and evaluated the humoral Th1/Th2 response in the combination of the adjuvants alum and CpG-ODN. In another experiment, to study the effect of the sensitization against orally fed antigens after DPT vaccination, we examined the specific antibody response to intragastrically administered antigen.

2. Materials and methods

2.1. Mice

Female BALB/c mice (3 weeks of age) were obtained from Japan SLC (Shizuoka, Japan). The animals were kept

in specific-pathogen-free conditions, in accordance with the standards approved by the Institutional Animal Care and Use Committee of Yokohama City University School of Medicine.

2.2. Oligonucleotide

Two types of phosphorothionate-modified ODNs were custom-synthesized and purified with HPLC by Sawady Technology (Kyoto, Japan). The following sequences were employed: the sequence under examination, ODN#1826, which is 5'-TCCATGACGTTTCCTGACGTT-3', and ODN#1911, which is a non-CpG DNA that was used as the control because it has a similar composition: 5'-TCCAGGACTTTCCTCAGGTT-3' [27–29].

2.3. DPT vaccine

Complete DPT vaccine (DPT-alum) and that without aluminum hydrate (DPT-non) were kindly given by Takeda (Tokyo, Japan). DPT-alum contains diphtheria and tetanus toxoids and pertussis toxin, filamentous hemagglutinin (FHA), aluminum hydrate (0.2 mg/ml) as adjuvant and thimelosal (less than 0.01%; w/v) as preservatives and also vaccine prepared with CpG; DPT-alum/ODN (DPT-alum with CpG-ODN 100 μ g/ml) and DPT-non/ODN (DPT without aluminum hydrate, but with CpG-ODN 100 μ g/ml).

2.4. Experimental schedule

In the first experiment, animals were injected intraperitoneally with 0.2 ml of DPT vaccine at day 0 (3 weeks of age), day 7 and day 14. Blood was taken from an orbital vein under anesthesia on days 6, 20 and 34. Sera were collected and stored at -80°C until analysis.

In the second experiment, mice were anesthetized and administered 0.5 ml of ovalbumin (OVA) in PBS (2 mg/ml) by gastric intubation with a 3 Fr indwelling feeding tube for infants (Atom Medical Co., Tokyo, Japan) with gentle handling on days 1, 3, 5, 8, 10, 12, 15, 17 and 19.

2.5. Immunoglobulin titration

Total IgE in the serum was measured using a mouse Yamasa IgE sandwich ELISA kit (Yamasa Co., Chiba, Japan) according to the manufacturer's instructions. Briefly, sera were diluted 1:25 and added to 96-well plates coated with anti-mouse IgE and incubated for 1 h. After washing, the total IgE was determined using horseradish peroxidase-conjugated anti-mouse IgE antibody and the plates were incubated for 1 h. After the plates were washed, they were developed using tetramethylbenzidine (TMB) solution, the reaction was stopped after 15 min with stop solution, and the OD was measured at 450 nm. The total IgE concentrations were determined from standard curves generated by serial dilution of a standard solution.

Anti-PT and anti-FHA specific IgG were measured with a Takeda ball ELISA kit (Takeda, Tokyo, Japan). Horseradish peroxidase-conjugated mouse anti-IgG monoclonal antibody was used as the second antibody, and the OD was measured at 405 nm. Blood samples for PT specific IgG1 and IgG2a were diluted and assayed by enzyme-linked immunosorbent assay (ELISA) using 1 µg/well of isotype-specific secondary antibodies (biotin-conjugated rabbit anti-mouse IgG1 or IgG2a; Cortex Biochem Inc., San Leandro, CA), and avidin-peroxidase conjugate (Sigma, Saint Louis, MO). These plates were developed using a TMB-1 component microwell substrate (KPL, Gaithersburg, MD) to determine the titration endpoint. The method for comparing Ig levels is an endpoint dilution, the titer being determined by the last dilution at which activity is detectable. The concentration of the PT antigen used to coat the plates was 10 µg/ml. OVA specific IgG1 and IgG2a titers were determined by ELISA using 1 µg/well, and were expressed in arbitrary units (au). Blood taken from mice injected i.p. three times with 0.5 mg OVA absorbed in alum (0.2 mg/ml) was pooled and the OVA-specific antibody titers were determined to be 2.56×10^6 au/ml for IgG1 and 6.4×10^3 au/ml for IgG2a as internal controls.

The OVA-specific IgE titer was measured by fluorometric reverse ELISA as previously described [30]. Briefly, plates were coated with 1 µg/ml anti-mouse IgE (Yamasa Co., Chiba, Japan) in the presence of bovine serum albumin (BSA) in 0.05 M carbonate–bicarbonate buffer (pH 9.5) for 3 h. After the wells were washed with PBS containing 0.05% Tween 20, mouse serum was added to duplicate wells and incubated overnight at room temperature, and then, after a second washing, 0.5 µg/ml biotinylated OVA was added to each well and the mixture incubated for 1 h at room temperature. The wells were washed a third time, and β-D-galactosidase-conjugated streptavidin (Boehringer-Mannheim, Germany) was added and incubated for 1 h at room temperature. After a further washing, 0.1 mM 4-methylumbelliferyl-β-D-galactoside was added to each well and incubated for 2 h at 37 °C. The reaction was stopped with 0.1 M glycine–NaOH (pH 10.3), and the fluorescence intensity was read from a microplate fluorescence

reader. OVA-specific antibody titers were determined to be 1.02×10^5 au/ml for IgE. Antibody titers were expressed by reference to the internal control.

2.6. Statistical analysis

Values are presented as the mean ± S.E.M. The statistical significance of differences between control and treated animals was estimated using Student's *t*-test. $p < 0.01$ was considered significant.

3. Results

3.1. Protective antibody production influenced by adjuvant in DPT vaccine

After intraperitoneal injection of DPT preparations in mice with or without adjuvant, alum and ODN, to evaluate the antibody response, anti-PT IgG antibody and anti-FHA antibody, the protective antibodies for pertussis, were determined by ELISA. As shown in Table 1, all the preparations of DPT, irrespective of any adjuvant additives, caused mice to produce antibodies against PT and FHA of pertussis against DPT immunization.

3.2. PT-specific IgG1 and IgG2a antibody responses after ODN-incorporating DPT vaccine administration

To determine the role of adjuvants in producing antibodies and to address the question whether the induction of antibody production depends upon the effects on Th1 cells or Th2 cells driven by DPT either conjugated or not conjugated with adjuvant, a differential assessment of PT-specific IgG1 and IgG2a titers was made (Fig. 1). Antibodies consisting more of the IgG1 than of the IgG2a subclass were determined after DPT-alum administration, and the equivalent amounts of antibodies of both IgG1 and IgG2a subclasses were demonstrated after DPT-alum/ODN injection and DPT-non/ODN injection, suggesting that IgG1 antibody production was not influenced by these adjuvants, and that alum and ODN adjuvant acted as strong inducers of the IgG2a response because the antibody titers of the IgG2a subclass against DPT-non/ODN and DPT-alum/ODN were increased by significantly more—about 100 times more—than those against DPT-non and DPT-alum ($p < 0.01$). This finding also suggested that DPT itself has a strong immunogenicity for IgG1 production, because IgG1 antibody titers were equally increased irrespective of the presence of alum adjuvant.

3.3. Serum total IgE level after ODN-containing DPT vaccine administration

Since the activation of Th2 cells eventually increases IgE production as well as IgG1 subclass antibody production,

Table 1

Protective antibody (anti-PT and anti-FHA IgG) titers in serum after vaccination with DPT-alum, DPT-non, DPT-alum/ODN and DPT-non/ODN (day 34)

	Anti-PT IgG	Anti-FHA IgG
PBS	—	—
DPT-alum	+	+
DPT-non	+	+
DPT-alum/ODN	+	+
DPT-non/ODN	+	+

Each antibody titer was obtained using a Takeda Ball ELISA kit. Plus (+) and minus (—) in this table indicate the presence and absence of protective antibodies, respectively. The experiment was performed three times. PT: pertussis toxin, FHA: filamentous hemagglutinin, alum: aluminum hydrate, ODN: oligodeoxynucleotide (#1826), DPT-alum: ordinary diphtheria–pertussis–tetanus vaccine, DPT-non: ordinary DPT vaccine without alum, DPT-alum/ODN: ordinary DPT vaccine with ODN, DPT-non/ODN: DPT-non with ODN.

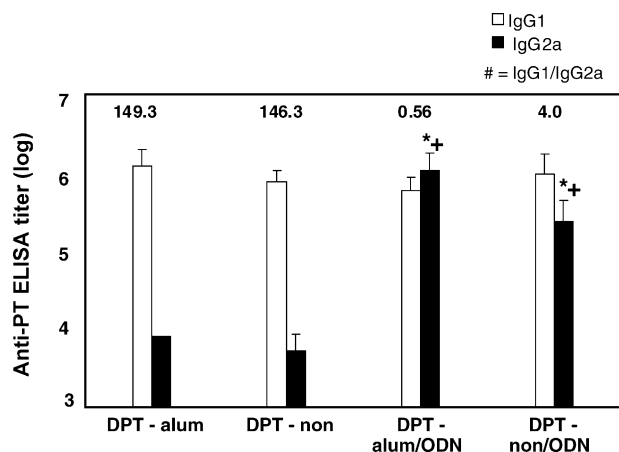


Fig. 1. Changes due to ODN in anti-PT titers. ODN induced a Th1-like response to DPT vaccine. Anti-PT antibody subclasses (IgG1 and IgG2a) in serum were measured after three administrations of DPT-alum, DPT-non, DPT-alum/ODN and DPT-non/ODN (day 34). Each column represents the group mean ($n = 4$) for anti-PT IgG1 and IgG2a antibody titers as determined by end-point dilution ELISA assay. Vertical lines represent the S.D. The numbers above each bar indicate the ratio of IgG1/IgG2a anti-PT antibody titer. One analysis typical of three individual experiments is shown. (*) $p < 0.01$ as compared with DPT-alum treated mice; (+) $p < 0.01$ as compared with mice treated with DPT-non. Student's t -test was used for statistical analyses.

we next examined the total IgE levels in the serum after DPT administration with or without alum and ODN. The total serum IgE levels were increased 3 and 5 weeks after the administration of DPT-alum, but not of DPT alone or DPT-non/ODN (Fig. 2). DPT-alum/ODN administration induced a slight increase of total IgE antibody production but the levels were significantly lower than those resulting from DPT-alum administration. These data indicate that the addition of ODN to DPT-alum had an inhibitory effect on the IgE antibody production activity of DPT-alum, and that ODN raised IgG2a and IgE production. Control ODN had no significant effect on the DPT-alum or DPT-non experimental groups (data not shown).

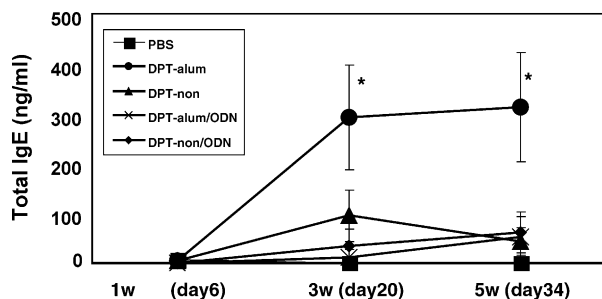


Fig. 2. Kinetics of induction of total IgE antibody in serum following vaccination with PBS, DPT-alum, DPT-non, DPT-alum/ODN and DPT-non/ODN. Each column represents the mean titer for 4–5 animals taken at the designated time. Vertical lines represent the S.D. The results of one analysis typical of five individual experiments are shown. (*) $p < 0.01$ as compared with mice treated with DPT-non, DPT-alum/ODN and DPT-non/ODN. Student's t -test was used for statistical analyses.

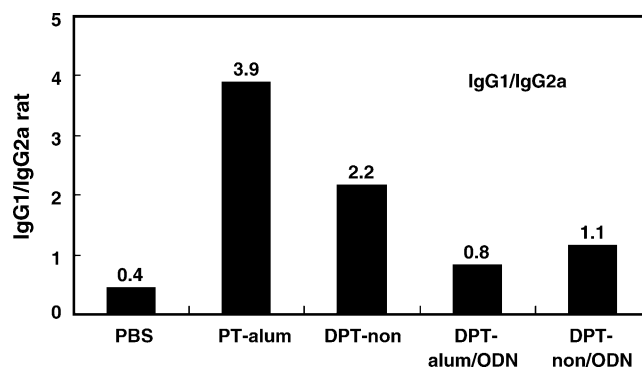


Fig. 3. ODN-induced Th1-like responses to orally administered OVA after DPT-alum, DPT-non, DPT-alum/ODN and DPT-non/ODN administration (day 34). Each column indicates the ratio of anti-OVA IgG1/IgG2a antibody titer. The numbers above each bar indicate the ratios. One analysis typical of three individual experiments is shown.

3.4. Antigen-specific IgG1 and IgG2a responses to orally administered antigen after ODN-containing DPT vaccine administration

When children are vaccinated with DPT, alum is administered simultaneously as an adjuvant to enhance the immunogenicity of the DPT vaccine. However, the antigens that will be exposed to the adjuvant are not always the expected vaccine proteins, but foreign antigens circulating in the blood, such as the proteins absorbed from the gastrointestinal tract. To investigate the action of an adjuvant upon these proteins, OVA was administered intragastrically to mice, which were then given commercially available DPT-alum vaccine intraperitoneally, and the antibody response to OVA was studied.

The ratio of IgG1 to IgG2a antibody titer was calculated to evaluate the Th1/Th2 immune response to administered vaccine (Fig. 3). This ratio decreased in ODN-treated groups. The IgG1-to-IgG2a ratio of the DPT-alum treated group was about five times that of the DPT-alum/ODN group.

These data suggest that DPT vaccine induces a Th2 type immune response with the help of added alum or PT toxin, but co-administration of ODN with DPT vaccine counterbalances the Th1/Th2 response to the administered vaccine.

3.5. Antigen-specific IgE response to orally administered OVA after ODN-containing DPT vaccine administration

To study the Th2 type immune response induced by the vaccine adjuvant after oral administration of antigen, we next observed antigen-specific IgE production of orally administered OVA (Fig. 4). OVA-specific IgE production was induced in the DPT-alum and DPT-non administered groups. On the other hand, in the group administered ODN-conjugated vaccine, no OVA-specific IgE production was induced ($p < 0.01$). These data suggest that ODN in vaccine inhibits IgE production against orally administered antigen.

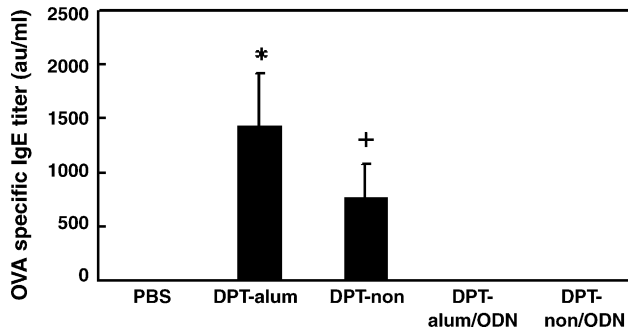


Fig. 4. Change in OVA-specific IgE titer determined by Sandwich ELSIA. ODN inhibits the IgE response to orally administered OVA (day 34). Each column represents the group mean for IgE titer to OVA. Vertical lines represent the S.D. One analysis typical of three individual experiments is shown. (*) and (+) $p < 0.01$ compared with mice treated with DPT-alum/ODN and DPT-non/ODN, respectively.

4. Discussion

Our studies demonstrated that adjuvants in ordinary DPT vaccine enhanced IgE antibody production in serum. In contrast, ordinary DPT vaccine with CpG-ODN (DPT-alum/ODN) enhanced anti-PT IgG2a antibody production by about one hundred times and reduced total IgE production in the serum in comparison with the changes induced in ordinary DPT (DPT-alum) administered mice. Moreover, DPT-alum/ODN decreased the ratio of IgG1/IgG2a that represents the Th2/Th1 balance of immune responses, and also reduced specific IgE production in response to orally administered antigen after vaccine administration.

Alum has been widely used as an adjuvant in both veterinary and human vaccines for nearly a century. It absorbs the vaccine ingredients and creates at the injected site a deposit from which antigen is slowly released. Also, alum activates both the antigen-presenting cells (APC) such as DC and macrophages, and complement, and induces humoral protective immune responses against infectious agents [31]. Although alum is efficient in inducing a strong antibody response, comparative studies have shown that it is a weak adjuvant for the induction of antibodies to protein subunits and for cell-mediated immunity [8], and it induced a much stronger Th2 pattern immune response in young animals than in adult animals [32]. PT is a major vaccine component and a virulence factor of *B. pertussis*, as well as an adjuvant in DPT vaccine. It has been shown that, in many animal models, PT directly stimulated the immune system, increased the Th2 response, and enhanced IgE production. IgE production is thought to depend on the ability of PT to upregulate IL-4 production, but the reason for this has not been elucidated at the molecular level. The toxin also enhances the expression of co-stimulatory molecules on macrophages, B cells and T cells [33], which presumably augments adjuvant effects. Although PT itself leads the Th2 immune responses, Th1 responses are necessary to prevent *B. pertussis* infections because *B. pertussis* can occupy an intracellular niche through invasion of, and survival in, alveolar macrophages

and other cells [34–36]. These immune responses against intracellular pathogens are mediated by Th1 cells, which activate phagocytes and facilitate the formation of opsonizing antibodies in a murine infection model [37]. A proper balance between the Th1 and Th2 protective immune responses is essential in vivo. However, in young children, the immune responses are different from those in the adult because of the immaturity of the immune system, and they tend to show Th2 responses rather than Th1 responses to antigens [38]. Thus, the vaccine antibody isotypes induced in infancy differ from those elicited later in life. Infant responses are characterized by a strong predominance of IgM, IgA and IgG1 responses, whereas the generation of IgG2 antibodies remains weak during the first 12–18 months of life, irrespective of the antigen or delivery system used. A reduced IgG2/IgG1 ratio could limit protection against infectious agents that require optimal complement binding for bacterial clearance [39].

CpG-ODN exerts strong stimulatory effects on the innate immune system, causing the secretion of inflammatory cytokines and inducing a Th1-biased immune response, including direct stimulation of APC through a toll-like receptor (TLR) 9 [40]. Thus, CpG-ODN acts as a potent adjuvant, with the result that some studies have employed CpG-ODN for more successful immunization in animal models and in non-human primates [19,23,41] and even in very young mice than an HbsAg-expressing DNA vaccine [23]. Also, used as an immune modulator for the prophylactic and therapeutic effects of CpG in allergies, CpG-ODN has been shown to downregulate IgE and Th2 cytokine production [42–44] in mouse models of asthma [45] and in human peripheral blood mononuclear cells (PBMCs) from atopic patients [46,47].

In this experiment, in consistency with many studies that used CpG-ODN as a vaccine adjuvant, ODN-conjugated ordinary DPT vaccine induces more than a hundred times the IgG2a titer to PT antigen. Also, we found that ordinary DPT vaccine administration increased IgE production, and that alum was responsible for inducing the IgE response. In combination with alum and CpG, IgG2a antibody induced both Th1 and Th2 responses without any reduction of the level of IgG1 induction. These results suggest that the co-delivery of CpG-ODN and alum in DPT vaccine is a more effective approach to successful immunization than the use of CpG-ODN or alum alone. The reason for this is that CpG-ODN is a small molecule consisting of short nucleic acid sequences, and can be easily disintegrated in vivo. Alum has the property of absorbing antigens, so CpG-ODN is more stable and effective when administered with alum in vivo [29]. However, in this mouse model, despite the fact that DPT vaccine induces Th2 responses, not only anti-PT IgG1 levels but also IgG2a levels increased after DPT vaccine administration in this study. The reason for this is presumably that, although PT itself has the potential of stimulating mainly a Th2 response, it also has some ability to stimulate a Th1 response [48].

DPT-alum vaccination and intragastric administration in mice led to the production of high titers of OVA specific IgE. However, no specific IgE production was seen after intragas-

tric administration of antigens in the control group. This may have been because the antibody responses to orally administered antigen absorbed via the gastrointestinal tract were affected by Th2 cytokines such as IL-4 and IL-13 induced by the alum or PT in DPT vaccine [5,46]. This result suggested that the Th1-biased immune responses to ODN counterbalanced the Th2 immune response after DPT administration. In the DPT-alum/ODN group, the IgG2a titer observed was double that in the DPT-non/ODN group. In this animal model, the control mice given both PBS by injection and oral OVA produced OVA IgG1 and IgG2a antibodies. We expected that immunological tolerance to orally administered antigens would be induced, but at the dose used and at the age of these mice, IgG antibody responses were induced, and the IgG2a production was greater than the IgG1 production.

In summary, in this study, we examined the efficiency of CpG-ODN in DPT vaccine and observed its effectiveness in combinations with alum in inducing protective immune responses to infectious agents. Also, CpG-alum/ODN vaccine counterbalanced Th2-dominant immune responses after DPT vaccine administration. The side effects of CpG DNA oligonucleotide administration have not been reported at this dose. In these ways, we believe that CpG-ODN with DPT vaccine contributes greatly to the successful and safe immunization of young children.

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