
Effect of sublingual administration with a native or denatured protein allergen and adjuvant CpG oligodeoxynucleotides or cholera toxin on systemic T_H2 immune responses and mucosal immunity in mice

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Background: Sublingual immunotherapy has been recently used for allergic diseases, but its mechanisms are still unclear.

Objective: To examine the effect of sublingual administration of a native or denatured allergen alone or plus adjuvant on systemic T_H2 responses and mucosal immunity in mice.

Methods: Naïve or sensitized BALB/c mice were sublingually vaccinated biweekly for 3 weeks with ovalbumin (OVA) or urea-denatured OVA (CM-OVA) only or plus adjuvant CpG oligodeoxynucleotides (CpG) or cholera toxin (CT). Two weeks later, their specific serum IgG, IgG1, IgG2a, IgE, and saliva secretory IgA (SIgA) antibody responses and the cytokine profiles of spleen and cervical lymph node cells were investigated.

Results: Specific SIgA antibody responses were induced by vaccination with CM-OVA plus CpG or CT. Whereas vaccination with CM-OVA and CpG enhanced T_H1 responses but inhibited IgE production, vaccination with CT and CM-OVA or OVA increased cervical lymph node cell production of interleukin (IL) 4, IL-5, and IL-6 and serum IgG1 antibody responses. In previously sensitized mice, sublingual vaccination with OVA or CM-OVA plus CT or CpG stimulated mucosal SIgA antibody responses, but did not enhance ongoing IgE antibody responses.

Conclusions: Sublingual vaccination with OVA or CM-OVA plus adjuvant CT or CpG all can induce systemic and mucosal immunity, but CM-OVA plus CpG had the best prophylactic and therapeutic effects on IgE antibody production. It is likely that sublingual vaccines may have a role for the prophylaxis and immunotherapy of allergic reactions.

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INTRODUCTION

Oral and intranasal administration of a native protein antigen to naïve adult animals can induce immune tolerance to the subsequent systemic challenge of the same antigen. This phenomenon has been documented as “mucosal tolerance.”^{1–3} Although the effect of sublingual administration of protein antigens to naïve animals has not been studied, to our knowledge, sublingual immunotherapy (SLIT) has been recently

used for the treatment of allergic diseases, such as allergic rhinitis and asthma.^{4,5} The underlying mechanisms of SLIT are still unclear.^{6–8} Furthermore, there is no animal model for sublingual vaccination.

Denaturation of ovalbumin (OVA) can markedly minimize its allergenicity,⁹ and oral administration of urea-denatured OVA (CM-OVA) can abrogate the induction of oral tolerance in mice.¹⁰ Because mucosal tolerance may play a crucial role in hindering the development of mucosal vaccination for a soluble protein antigen,¹¹ its denatured particulate form may abolish mucosal tolerogenicity and, thus, can grant the best efficacy of mucosal immunization. Therefore, the influence of sublingual administration of a denatured protein vaccine on mucosal and systemic immune responses will be examined for the first time, to our knowledge, in the present study. It is also interesting to further compare the sublingual vaccination effects between native and denatured protein vaccines.

After intranasal immunization with a protein antigen and mucosal-adjuvant cholera toxin (CT) or oligodeoxynucleotides containing immunostimulatory CpG motif (CpG), mucosal and systemic antibody responses are significantly induced.^{12–15} In the present study, we will further elucidate the

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effects of sublingual vaccination with a denatured particulate allergen mixed with CpG or CT on specific mucosal and systemic immunity. To our knowledge, we have demonstrated for the first time that sublingual vaccination with a denatured allergen plus CpG effectively enhances mucosal secretory IgA (SIgA) responses and systemic T_H1 responses and suppresses ongoing IgE antibody responses.

METHODS

Animals

Female BALB/c mice (aged 6–8 weeks) were obtained from the National Animal Center of Taiwan, Taipei. They were maintained on a standard diet (Lab diet; PMI Feeds, St Louis, Missouri) in the Animal House of Taipei Veterans General Hospital, Taipei. All the experiments were performed at least twice.

Antigen Preparation

Ovalbumin (grade 5) was obtained from Sigma, St Louis. Denaturation of OVA was performed as described previously.¹⁰ Briefly, CM-OVA was prepared by reduction of disulfide bonds after stirring overnight under vacuum suction with 8.0M urea and 0.2M 2-mercaptoethanol (Merck, Darmstadt, Germany). Carboxymethylation of CM-OVA was prepared by alkylation of the sulfhydryl groups of CM-OVA after stirring for 3 hours in the dark with 0.3M sodium iodoacetate (Sigma) at pH 8.0. The crude preparation of CM-OVA was extensively dialyzed against distilled water and then lyophilized.

Adjuvants and Sublingual Administration of Antigen

A total of 20 base phosphorothioate-modified oligonucleotides were synthesized (CpG) (Sigma): 5'-TCCATGACGT-TCCTGACGTT-3'. Both CpG and CT (Calbiochem, San Diego, California) were used as adjuvants.

Groups of 6 to 8 BALB/c mice were sublingually administered 100 µg of OVA or CM-OVA mixed with 10 µg of CpG or 1 µg of CT in 10-µL isotonic sodium chloride solution (saline) biweekly for 3 weeks. Control mice were treated with saline only. All the mice were lightly sedated with 1 mg of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, Illinois) before treatment.

Systemic Sensitization

Mice were sensitized intraperitoneally with 10 µg of OVA adsorbed onto 4 mg of aluminum and suspended in 0.5 mL of phosphate-buffered saline (PBS). A blood sample was obtained from the tail, and their saliva was collected after intraperitoneal injection of carbamylcholine chloride, 10 µg per mouse (Sigma).

Experimental Protocol

In experiment 1, naïve mice were sublingually vaccinated biweekly for 3 weeks with OVA, CM-OVA, or saline, only, or plus adjuvant CpG or CT. Two weeks later, they were sensitized. Their cytokine profiles of spleen and cervical lymph node (CLN) cells and specific serum and saliva anti-

body responses were evaluated before sensitization or 2 weeks after sensitization. In experiment 2, mice were first sensitized. Two weeks later, they were sublingually vaccinated biweekly for 3 weeks, as previously described. Their specific serum and saliva antibody responses were evaluated 2 weeks after vaccination (Table 1).

Evaluation of Antibody Responses

Antibody levels were tested by enzyme-linked immunosorbent assays, as previously described.¹⁶ Briefly, 96-well plates (Nunc, Kamstrup, Roskilde, Denmark) were coated with 100 µg/mL of OVA or 10 µg/mL of goat antimouse IgA (Sigma) in 0.05M carbonate buffer, pH 9.6 (100 µL per well), overnight at 4°C. Free sites were blocked with 3% skimmed milk in PBS–polysorbate 20 (Tween 20) for 1 hour. Saliva or serum samples (1/100 to 3000) and standards (mouse IgA, Sigma; or pooled hyperimmune serum samples after monthly treatment with OVA or complete Freund adjuvant) were added in duplicate, and the plates were incubated for 5 hours at room temperature. After washing, 100 µL of horseradish peroxidase–conjugated goat antimouse IgA (1/4000; Sigma), IgG (1/4000; Jackson, West Grove, Pennsylvania), or IgG2a or IgG1 (1/4000; SBA, Birmingham, Alabama) were added and the plates were incubated overnight at 4°C. After washing, orthophenylenediamine, 0.5 mg/mL (Sigma), in citrate-carbonate buffer containing 0.015% hydrogen peroxide, was added and the plates were incubated in the dark. Color

Table 1. Experimental Groups and Protocol^a

Group	Antigen	Adjuvant
Experiment 1		
1	OVA	None
2	OVA	CT
3	OVA	CpG
4	CM-OVA	None
5	CM-OVA	CT
6	CM-OVA	CpG
7	Saline	None
Experiment 2		
1	OVA	None
2	OVA	CT
3	OVA	CpG
4	CM-OVA	None
5	CM-OVA	CT
6	CM-OVA	CpG
7	Saline	None

Abbreviations: CM-OVA, denatured ovalbumin; CpG, CpG oligodeoxynucleotides; CT, cholera toxin; OVA, ovalbumin; Saline, isotonic sodium chloride solution.

^a For experiment 1, vaccination was performed during weeks 1 through 3 and sensitization was performed during week 5. Sample collection for cervical lymph node and spleen cells occurred during week 4; and for blood and saliva, during week 5 (before sensitization) and week 7 (after sensitization). For experiment 2, sensitization was performed during week 1 and vaccination was performed during weeks 3 through 5. Sample collection for blood and saliva occurred during week 7.

development was stopped by 4N sulfuric acid. Absorbance at 492 nm was read using a reader (SPECTRAmax 250; Molecular Devices, Sunnyvale, California), and unknowns were interpolated.

Cytokine Assays

One week after the last sublingual vaccination, CLN and spleen cells from 5 BALB/c mice were collected. The CLN and spleen cells (5×10^5 cells per well for CLN cells and 2×10^6 cells per well for spleen cells) from the same group were mixed and cultured in duplicate in complete Roswell Park Memorial Institute 1640 medium with OVA, 1 mg/mL, 10% fetal calf serum, and antibiotics in 24-well flat-bottomed microtiter plates, 1 mL per well (Costar, Cambridge, Massachusetts). The supernatants were harvested after 1 to 3 days of culture, and cytokines IL-4, IL-5, IL-6, and interferon- γ (IFN- γ) were measured using sandwich enzyme-linked immunosorbent assay kits (eBioscience, San Diego, California).

Passive Cutaneous Anaphylaxis

Specific IgE antibody responses were evaluated as described previously.¹⁷ Briefly, passive cutaneous anaphylaxis tests were examined in quadruplicate in Sprague-Dawley rats (300–450 g) obtained from the Animal Center, National Yang-Ming University, Taipei, Taiwan. Aliquots of 100 μ L of 2-fold dilutions of each pooled mouse serum sample (1/50–1/1600) were intradermally injected onto 4 rats. They were challenged 48 hours later by an intravenous injection of 2 mg of OVA and 5 mg of Evans blue in 1 mL of PBS. The reaction was read 30 minutes later. The passive cutaneous anaphylaxis titer was the highest dilution, giving a positive reaction of at least 5 mm in diameter, and was expressed as mean \pm 1 SEM.

Statistical Analysis

Antibody and cytokine titers were expressed as mean \pm 1 SD, and all group comparisons for specific serum IgG, IgG1, and IgG2a and saliva SIgA antibody responses were made using 2-tailed Wilcoxon rank sum tests.

RESULTS

Effect of Sublingual Vaccination on Systemic Antibody Responses Before Sensitization

The effect of sublingual vaccination was first studied. Groups of BALB/c mice were sublingually vaccinated biweekly for 3 weeks with OVA or CM-OVA alone or together with CT or CpG. The control group was treated with saline only. Two weeks after the last treatment, their systemic immune responses were examined (Fig 1). The mice receiving CM-OVA and adjuvant CT or CpG had higher specific IgG antibody responses than those receiving native OVA and adjuvant CT or CpG ($P < .05$ and $P < .005$, respectively) (Fig 1A). Although those receiving CT had much higher IgG1 antibody responses (Fig 1B), the group receiving CpG had much higher IgG2a antibody responses (Fig 1C). Furthermore, the group receiving CM-OVA and CpG had much stronger IgG2a antibody responses than those receiving OVA and CpG ($P < .005$) (Fig 1C). In addition, sublingual

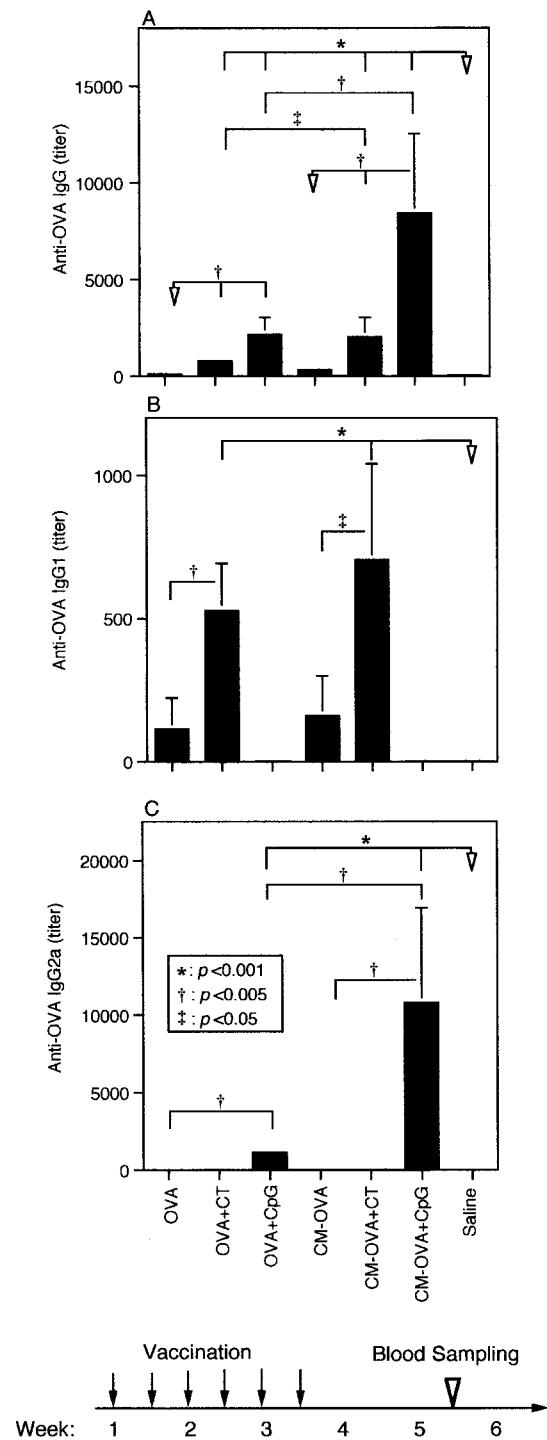


Figure 1. Effect of sublingual vaccination on specific systemic antibody responses. Groups of 6 to 8 BALB/c mice were sublingually vaccinated biweekly for 3 weeks with 100 μ g of ovalbumin (OVA) or carboxymethylation of denatured OVA (CM-OVA) only or plus 1 μ g of cholera toxin (CT) or 10 μ g of CpG oligodeoxynucleotides. Two weeks after the last treatment, their systemic specific antibody responses were examined for IgG (A), IgG1 (B), and IgG2a (C).

vaccination failed to induce an IgE antibody response, even in the groups receiving T_H2-dominant adjuvant CT (data not shown).

Effect of Sublingual Vaccination on Systemic Antibody Responses After Sensitization

The prophylactic effect of sublingual vaccination on T_H2 responses was further studied. Groups of mice were treated as

described in Figure 1. Two weeks after the last treatment, they were intraperitoneally sensitized. Their systemic immune responses were evaluated 2 weeks after sensitization (Fig 2). The mice receiving OVA or CM-OVA and CT or CpG had higher specific IgG antibody responses than the controls ($P < .001$) (Fig 2A). Although the groups receiving antigen plus CT had much higher IgG1 antibody responses

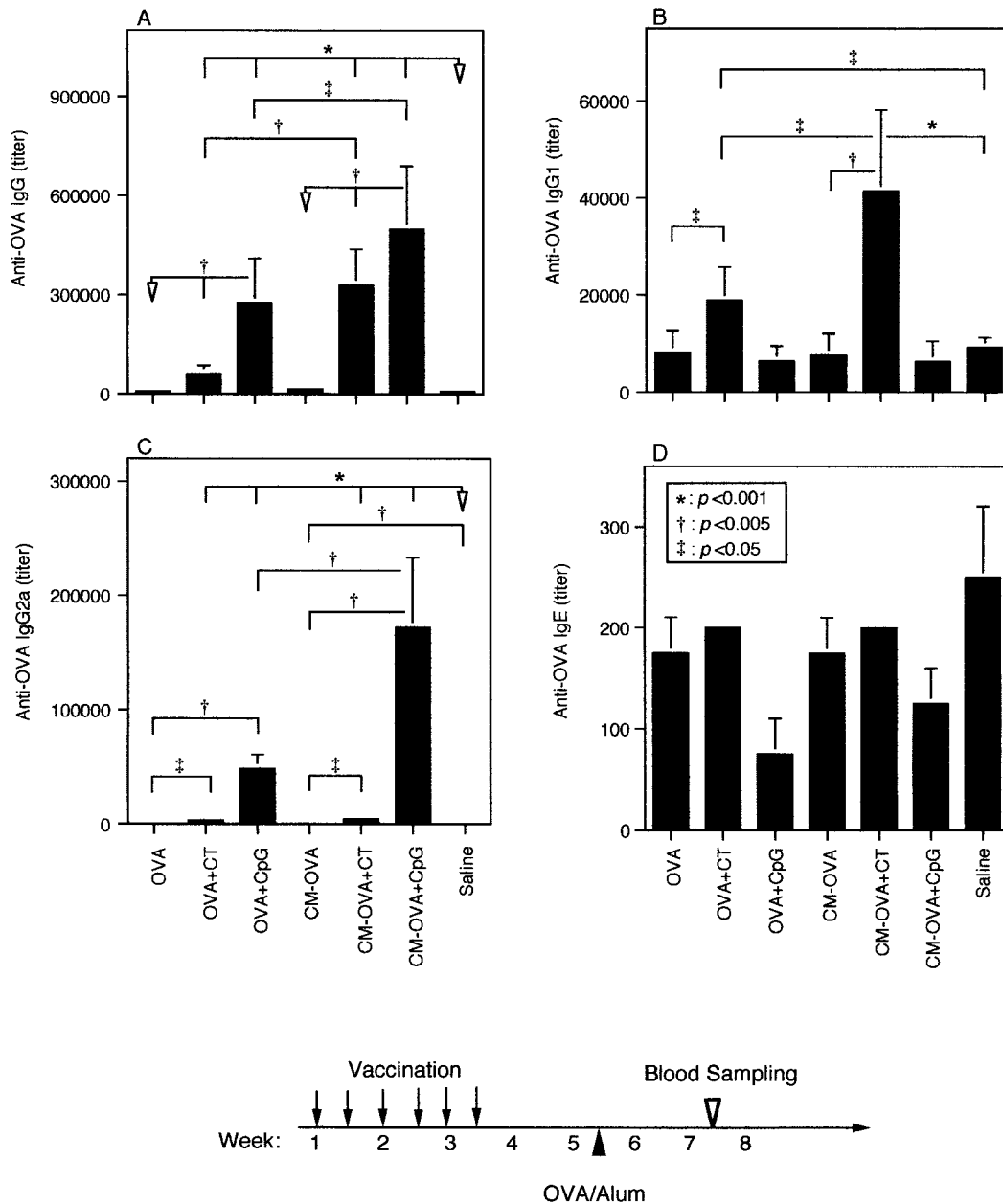


Figure 2. Effect of sublingual vaccination on specific systemic antibody responses after sensitization. Groups of 6 to 8 BALB/c mice were sublingually vaccinated as described in Figure 1. Two weeks after the last vaccination, they were sensitized. Their specific individual IgG (A), IgG1 (B), and IgG2a (C) and pooled IgE (D) antibody responses were examined 2 weeks after sensitization. CM-OVA indicates denatured ovalbumin; CpG, CpG oligodeoxynucleotides; CT, cholera toxin.

(Fig 2B), the groups receiving antigen plus CpG had much higher IgG2a antibody responses (Fig 2C). When compared with the group receiving OVA and CpG, those receiving CM-OVA and CpG had enhanced IgG2a antibody responses ($P < .005$) (Fig 2C). Similarly, the group receiving CM-OVA and CT also had enhanced IgG1 antibody responses when compared with those receiving OVA and CT ($P < .05$) (Fig 2B). After sensitization, all the groups developed specific IgE responses (Fig 2D). Sublingual vaccination with either antigen alone or antigen plus CT or CpG did not induce higher IgE responses than in the controls. In contrast, those receiving native or CM-OVA and CpG had more than a 50% decrease of IgE antibody responses than controls.

Effect of Sublingual Vaccination on Specific Mucosal Antibody Responses

The effect of sublingual vaccination on SIgA antibody responses was also studied. Groups of mice were treated as described in Figure 1 or Figure 2. Either before or after sensitization, the mice vaccinated together with CpG showed profoundly increased SIgA antibody responses when compared with the controls ($P < .001$) (Fig 3A and B). In addition, only those receiving CM-OVA and CT showed higher SIgA antibody responses than the controls ($P < .001$) (Fig 3A and B). The CpG had more potently adjuvant effects than CT on SIgA production (Fig 3A and B). Whereas specific IgG, IgG1, and IgG2a antibody responses were all enhanced after systemic sensitization (Fig 1 and Fig 2), specific SIgA production was not enhanced after sensitization (Fig 3A and B). Furthermore, sublingual vaccination did not affect total saliva SIgA levels (Fig 3C).

Effect of Sublingual Vaccination on Cytokine Secretion of CLN and Spleen Cells

The cytokine profiles after sublingual vaccination were further studied. Groups of 5 BALB/c mice were sublingually vaccinated as described in Figure 1. Their CLN and spleen cells were collected and mixed 1 week after the last treatment. Their cytokine profiles are shown in Figure 4 and Figure 5, respectively. Compared with the mice receiving OVA, those receiving CM-OVA showed enhanced cytokine production, including IFN- γ and IL-5 in CLN cells (Fig 4) or IFN- γ , IL-4, and IL-5 in spleen cells (Fig 5). Those receiving CT plus OVA or CM-OVA showed increased production of IL-4 in CLN cells (Fig 4B) and spleen cells (Fig 5B). In contrast, those receiving CM-OVA and CpG had weakly enhanced production of IFN- γ in CLN and spleen cells (Fig 4A and Fig 5A). In addition, those receiving OVA or CM-OVA plus CpG or CT had much enhanced IL-5 and IL-6 production in CLN cells (Fig 4C).

Therapeutic Effect of Sublingual Vaccination in Previously Sensitized Mice

The therapeutic effect of sublingual vaccination on ongoing T_H2 responses was further elucidated. Groups of mice were first intraperitoneally sensitized with OVA and alum. Two weeks later, they were sublingually vaccinated biweekly as

previously described for 3 weeks. Control mice were similarly treated with saline only. Their serum and saliva samples were collected 2 weeks after the last treatment. The mice receiving OVA or CM-OVA plus CpG or CT had higher saliva-specific SIgA antibody responses than the controls ($P < .005$) (Fig 6A). When compared with the mice treated with OVA or CM-OVA only, higher specific SIgA antibody responses were found when mucosal-adjuvant CT ($P < .05$ and $P < .005$, respectively) or CpG ($P < .05$ and $P < .005$, respectively) (Fig 6A) was added. When compared with those treated with native OVA plus CT or CpG, specific SIgA antibody responses were enhanced in those treated with CM-OVA plus CT or CpG ($P < .05$) (Fig 6A). When compared with the controls, those receiving CpG plus OVA or CM-OVA had much higher IgG2a antibody responses ($P < .005$ for both) (Fig 6C) and lower IgG1 antibody responses ($P < .005$ and $P < .05$, respectively) (Fig 6B). Similarly, the specific IgE antibody responses in the mice receiving T_H1-adjuvant CpG and OVA or CM-OVA had more than 50% inhibition than those in the controls (Fig 6D).

DISCUSSION

We have demonstrated herein that both mucosal and systemic immune responses can be simultaneously induced by sublingual vaccination with a protein antigen and mucosal-adjuvant CT or CpG. When compared with the native protein vaccine with or without adjuvant, the present study also revealed that the denatured protein vaccine with or without adjuvant has better efficacy in sublingual vaccination. Although CT and CpG are powerful mucosal adjuvants to trigger strong SIgA production, T_H1-dominant adjuvant CpG is better than T_H2-dominant adjuvant CT for inhibiting IgE production in sublingual vaccination.

Consistent with reports^{12,13,18,19} that both systemic and intranasal immunization with a protein antigen and adjuvant CpG can induce T_H1-predominant immunity, we have demonstrated for the first time, to our knowledge, that sublingual vaccination with either OVA or CM-OVA with adjuvant CpG also exclusively induces T_H1-predominant systemic immune responses. As determined before,^{12,18} this is characterized by serum strong IgG2a antibody reactions and by high levels of IFN- γ production in CLN and spleen cells. Adjuvant CT has been shown to predominantly stimulate T_H2-biased immune responses.²⁰ Recently, other studies^{21,22} also described that CT could enhance mixed T_H1 and T_H2 responses. In the present study, the mice receiving the protein antigen and CT had strong IgG1 but weak IgG2a antibody responses. Their CLN and spleen cells also secreted strong T_H2 (IL-4) but weak T_H1 (IFN- γ) cytokines. Consistent with reports¹²⁻¹⁵ that CT and CpG are potent intranasal mucosal adjuvants, our study further demonstrated that sublingual vaccination with adjuvant CT or CpG can significantly induce specific SIgA antibody responses. Whereas adjuvant CpG and CT enhanced specific systemic and mucosal immune responses, they did not affect serum total IgG1 and IgG2a antibody levels and

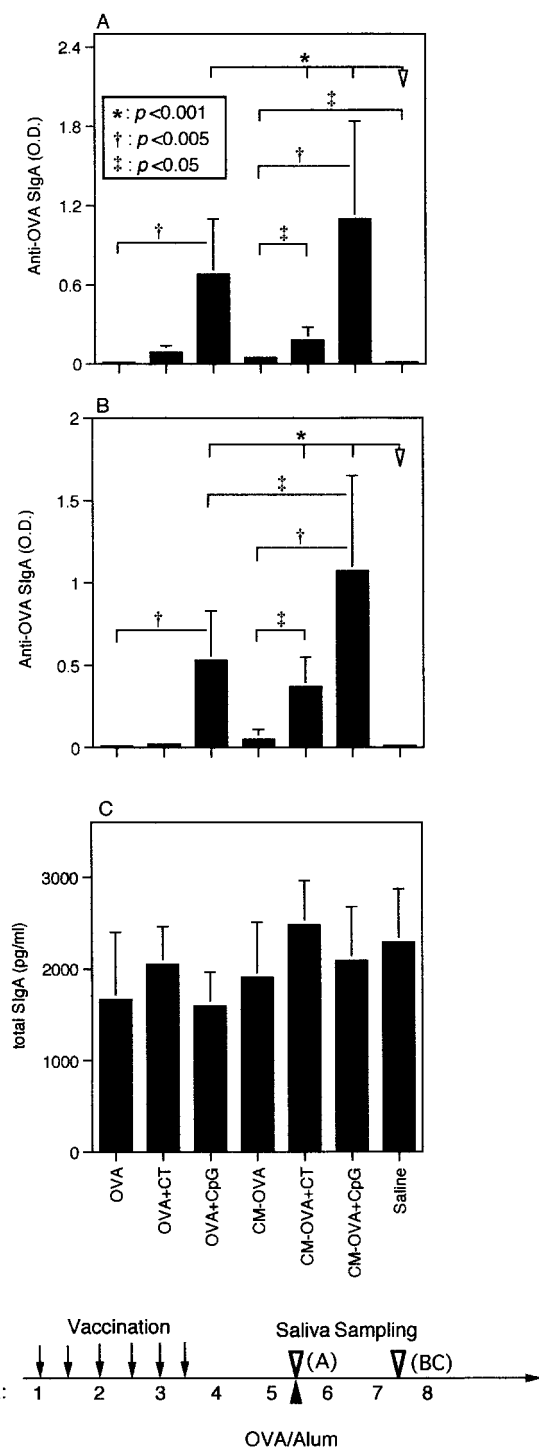


Figure 3. Effect of sublingual vaccination on specific mucosal antibody responses. Groups of mice were treated as described in Figure 1 or Figure 2. Their saliva secretory IgA (SIgA) antibody responses were examined 2 weeks after the last sublingual vaccination or 2 weeks after sensitization and shown as antiovalbumin (OVA) SIgA before sensitization (A), anti-OVA SIgA after sensitization (B), and total SIgA after sensitization (C). Abbreviations are expanded in the footnote to Figure 2.

circulating cytokines, including IL-4 and IFN- γ (data not shown).

Denaturation of the protein destructs its tertiary structure and leads to random-coiled aggregation and decreased solubility.^{23,24} The structure of a native protein plays a critical role for IgE-binding conformational epitopes.^{25,26} Although native OVA is a soluble protein, CM-OVA may be highly aggregated into the particulate form. Denaturation of OVA leads to 10-fold higher frequency of IFN- γ -producing cells than native OVA.²⁷ The particulate antigen may be mostly localized in the draining lymph nodes and is, thus, more efficiently processed by antigen-presenting cells. This implication is further confirmed by the present study, which shows that vaccination with CM-OVA with or without adjuvant induces higher systemic and mucosal antibody responses, characterized by strong serum IgG and saliva SIgA antibody levels.

The adverse effects of injection vaccines include soreness at injection sites and the possibility of lethal anaphylaxis. Furthermore, parenteral vaccination is not able to effectively induce mucosal immunity.²⁸ Therefore, vaccination through mucosal routes is a better choice to avoid the adverse effects of injection and to induce mucosal immunity. When protein antigens are orally administered, they may lose most of their antigenicity because of extensive hydrolysis by gastrointestinal enzymes.²⁹ To overcome this disadvantage, a large dosage of oral antigens may be needed for an oral protein vaccine in mice.⁹ Protein digestion begins in the stomach. Thus, protein antigens are still intact in the oral cavity. Theoretically, sublingual vaccines need a much lower dosage of proteins than oral vaccines. Indeed, sublingual vaccination with a low dosage of CM-OVA (100 μ g) can effectively induce systemic and mucosal antibody responses. The present finding suggests that sublingual vaccination is a rational strategy to reduce the cost of antigen preparation and the adverse effects of injection vaccines and to preferentially enhance specific mucosal and systemic immunity.

The prophylactic effect of sublingual vaccination on allergy was found in the groups receiving CpG and native or denatured OVA. Both had much enhanced T_H1 responses. After systemic sensitization, their specific IgE antibody responses were significantly lower than those of the control group. Furthermore, the therapeutic effects of sublingual vaccination on allergy were also disclosed in the previously sensitized groups. The mice sublingually treated with OVA or CM-OVA plus CpG had enhanced T_H1-modulated IgG2a antibody responses but decreased IgE antibody responses. When compared with the control group, higher specific SIgA antibody responses were induced by SLIT with OVA or CM-OVA plus adjuvant CpG or CT. The SIgA may effectively act as blocking antibodies to prevent allergen penetration into the mucosa (immune exclusion). Therefore, sublingual vaccination is possibly a rather safe and useful strategy for the immunotherapy of allergic disorders.

Intranasal or oral application of soluble proteins has been shown to induce regulatory T cells in animal models.^{2,30,31} For

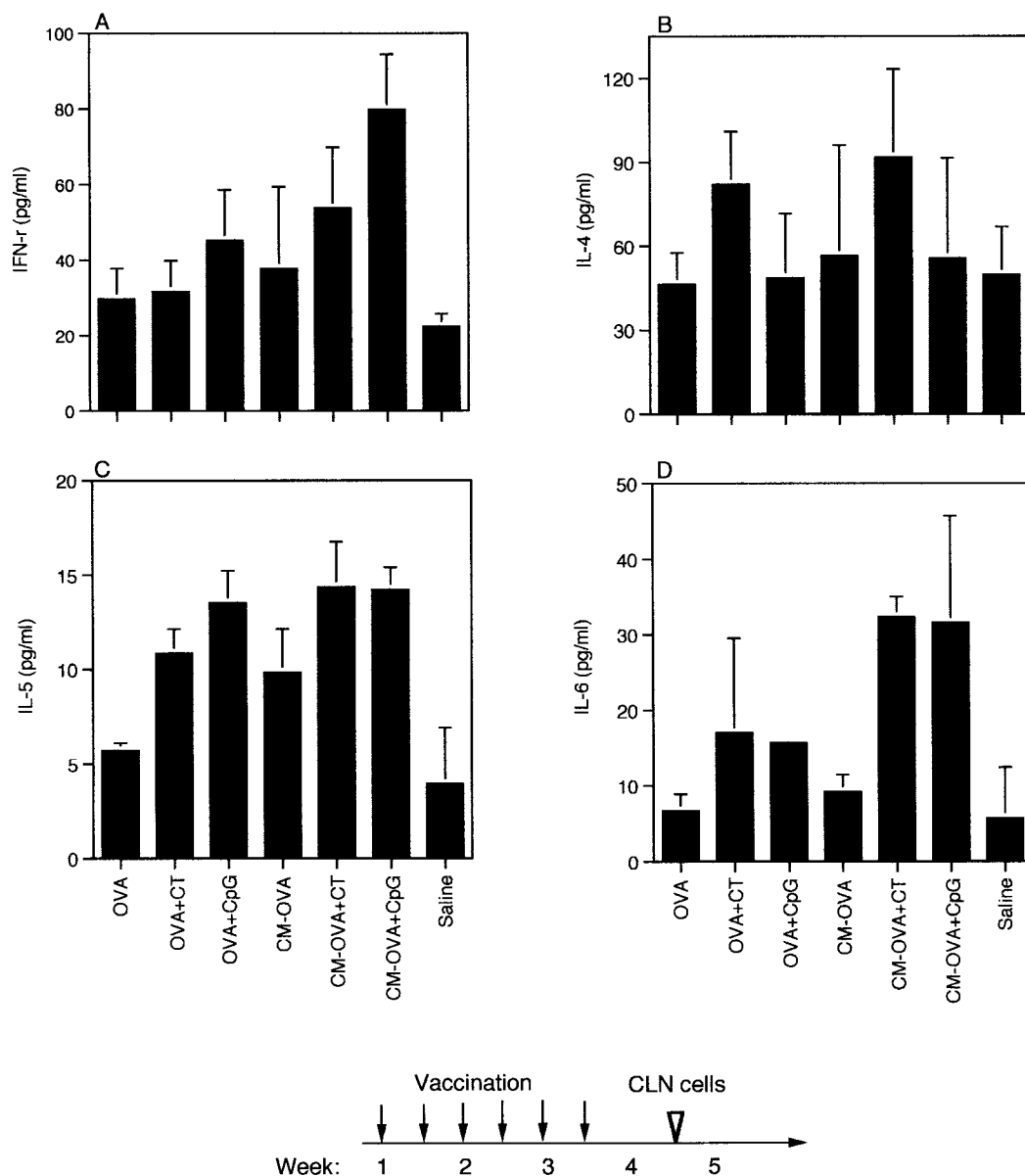


Figure 4. Effect of sublingual vaccination on cytokine secretion of cervical lymph node (CLN) cells. Groups of 5 BALB/c mice were sublingually vaccinated as described in Figure 1. Their CLN cells were collected 1 week after the last vaccination. The CLN cells (5×10^5 cells per well) from the same group were mixed and cultured with ovalbumin (OVA) in vitro for 1 or 3 days. The cytokines in the supernatants, including interferon- γ (IFN- γ) (A), interleukin (IL) 4 (B), IL-5 (C), and IL-6 (D), were measured in duplicate using enzyme-linked immunosorbent assay kits. Abbreviations are expanded in the footnote to Figure 2.

instance, intranasal administration of OVA in mice significantly prevented the subsequent specific IgE and IgG1 antibody responses.³¹ Whereas Barbey et al³¹ showed that intranasal administration of 2 mg of OVA to the mice had a better tolerizing effect, we failed to find any difference in the specific IgE and IgG1 antibody responses between the controls and the mice sublingually treated with 100 μ g of OVA. Our finding may suggest that sublingual administration of 100 μ g of OVA to mice cannot induce marked tolerance.

Whether sublingual administration of higher doses of OVA can lead to a tolerant effect is still unclear. Thus, the kinetics of different dosages of sublingual antigens need further investigation. Unger et al³ further showed that regulatory T cells could be induced in the mice receiving intranasal vaccination, but not in those undergoing intramuscular injection. Similarly, we found herein that the priming effect was readily induced by parenteral sensitization, but not by sublingual vaccination. Furthermore, it will be interesting to extensively

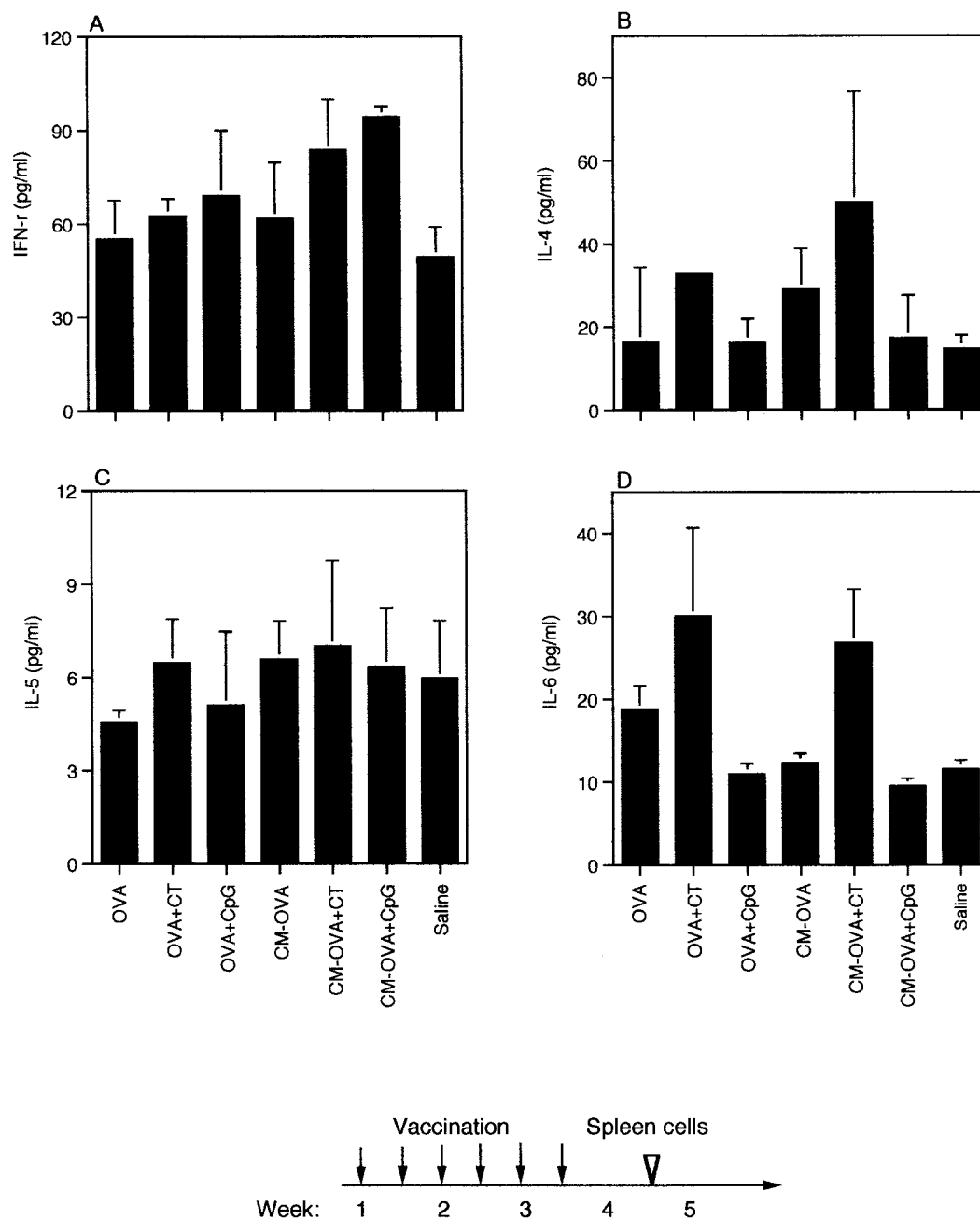


Figure 5. Effect of sublingual vaccination on cytokine secretion of spleen cells. Groups of 5 BALB/c mice were treated as described in Figure 4. Their spleen cells were collected 1 week after the last treatment. The spleen cells (2×10^6 cells per well) from the same group were mixed and cultured with ovalbumin (OVA) in vitro for 1 or 3 days. The cytokines in the supernatants, including interferon- γ (IFN- γ) (A), interleukin 4 (IL-4) (B), IL-5 (C), and IL-6 (D), were measured in duplicate using enzyme-linked immunosorbent assay kits. Abbreviations are expanded in the footnote to Figure 2.

compare the effects of protein allergens and adjuvants on specific IgE production through different pathways.

Our studies showed that sublingual vaccination with denatured allergen and adjuvant CpG had prophylactic and therapeutic effects on specific IgE production. In combination with mucosal-adjuvant CpG or CT, sublingual vaccination

with native or denatured OVA was able to enhance serum specific IgG antibody responses and saliva specific SIgA antibody responses. In conclusion, sublingual vaccination with a denatured allergen and mucosal-adjuvant CpG can induce mucosal SIgA antibody responses and suppress specific IgE production simultaneously.

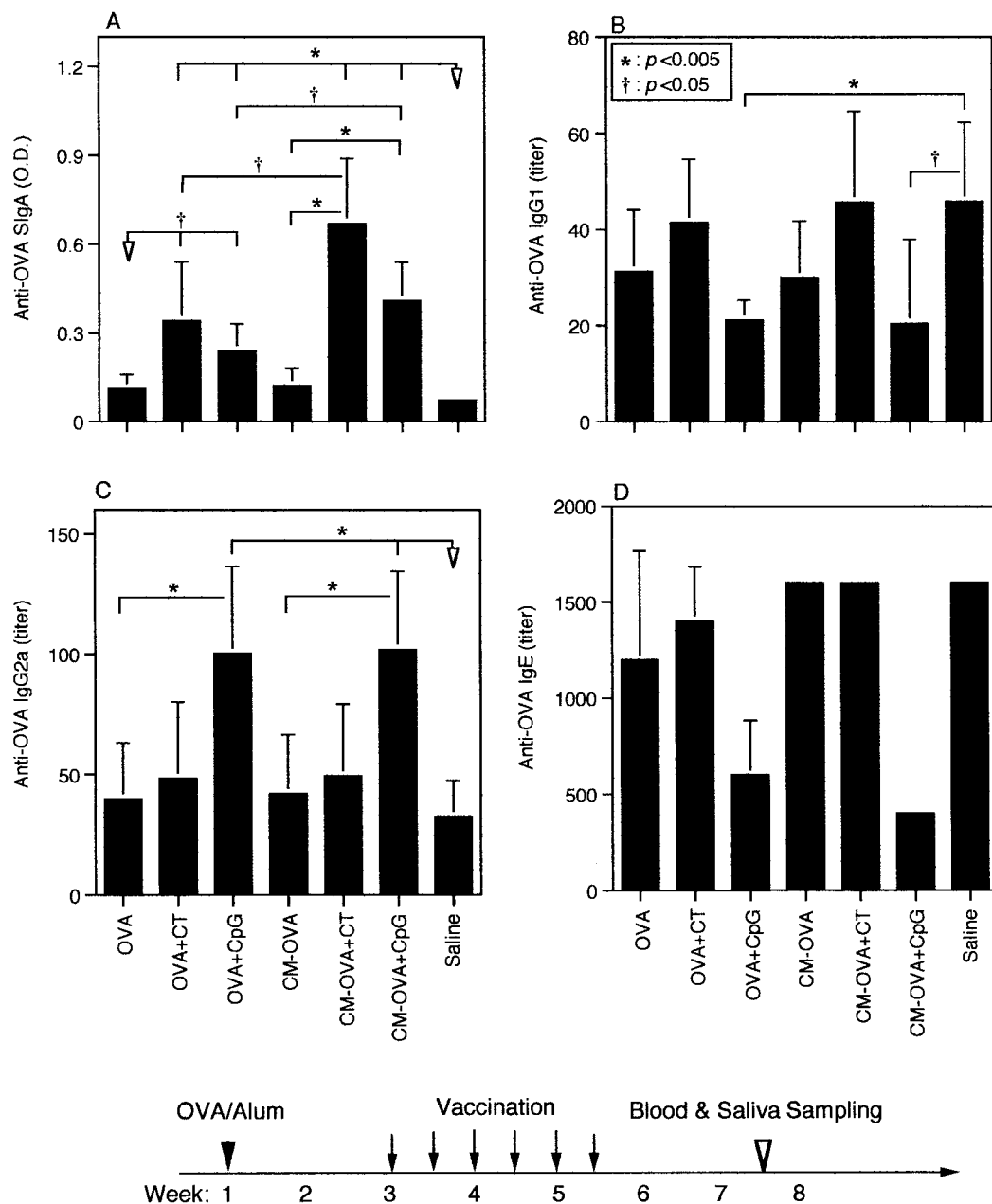


Figure 6. Therapeutic effect of sublingual vaccination in previously sensitized mice. Groups of 6 BALB/c mice were sensitized with ovalbumin (OVA) and alum. Two weeks after sensitization, they received sublingual vaccination biweekly for 3 weeks. Control mice were similarly treated with isotonic sodium chloride solution (saline) only. Two weeks after the last treatment, their specific individual IgG, IgG1, and IgG2a and pooled IgE antibody responses were analyzed and shown as saliva secretory IgA (SIgA) (A), serum IgG1 (B), serum IgG2a (C), and serum IgE (D). Abbreviations are expanded in the footnote to Figure 2.

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