

Effect of Neonatal Sublingual Vaccination with Native or Denatured Ovalbumin and Adjuvant CpG or Cholera Toxin on Systemic and Mucosal Immunity in Mice

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Abstract

Sublingual immunotherapy has been applied for allergic diseases, but whether sublingual immunization in neonates can prevent sensitization has not been studied. In this study, we evaluate the effect of neonatal sublingual vaccination with native or denatured allergens alone or plus adjuvant on allergy prevention. Newborn BALB/c mice were sublingually vaccinated daily for the first 3 days with native or denatured ovalbumin (OVA) only, or combined adjuvant CpG or cholera toxin (CT). They were sensitized with OVA adsorbed onto alum 7 weeks after the last vaccination. Specific secretory IgA antibody responses were readily induced by neonatal vaccination with antigen plus CpG or CT, but not with antigen alone. Whereas vaccination with denatured OVA plus CpG markedly enhanced T helper 1 (Th1) responses and inhibited IgE production, vaccination with denatured OVA plus CT increased cervical lymph node cell production of interleukin-4 (IL-4), IL-5, IL-6, and serum IgG1 responses. These data demonstrate that neonatal sublingual vaccination with denatured OVA and CpG not only preferentially induces systemic Th1 responses and mucosal immunity, but also simultaneously abrogates IgE production. Neonatal sublingual vaccines may play a role for the strategy of allergy prevention.

Introduction

Development of neonatal vaccines has been hindered by poor efficacy because of the immature immune system [1], but Ridge *et al.* [2] has demonstrated that neonatal T cells can be activated by professional antigen presenting cells. A recent study has also shown that neonatal immune responses can be induced by rectal administration with *Mycobacterium bovis* Bacillus Calmette-Guérin [3]. Sublingual immunotherapy (SLIT) has been recently applied for the treatment of allergic diseases such as allergic rhinitis and asthma [4, 5], but its mechanisms remain unclear [6, 7]. Furthermore, the effect of sublingual administration of protein allergens on newborn animals has not been elucidated.

After intranasal (i.n.) immunization with a protein antigen, both mucosal and systemic antibody responses can be simultaneously induced in newborn mice [3, 8]. Theoretically, they may be similarly induced after

sublingual administration with a protein antigen in newborn mice. We have previously reported that denaturation of ovalbumin (OVA) can destroy its conformational epitopes and thus markedly minimize its IgE-binding reactivity [9, 10]. On the other hand, we have also shown that oral administration of denatured OVA can abrogate the induction of oral tolerance of Th2 immune responses in adult mice [9]. Therefore, the effects of neonatal sublingual administration with denatured OVA on mucosal and systemic immune responses will be examined for the first time in the present study. It is also interesting to compare the influence of sublingual vaccination between native and denatured protein antigens.

In addition, it is interesting to further elucidate the effect of neonatal sublingual vaccination with native or denatured OVA combined with mucosal adjuvant such as oligodeoxynucleotides containing immunostimulatory CpG motif (CpG) or cholera toxin (CT) on specific

mucosal and systemic immunity. Indeed, we have demonstrated here for the first time that neonatal sublingual vaccination with a denatured OVA and adjuvant CpG effectively induces mucosal secretory IgA (SIgA) and systemic T helper 1 (Th1) cell responses and suppresses subsequent IgE antibody responses.

Materials and methods

Animals. Adult (10–12 weeks) BALB/*c* mice were obtained from the National Animal Center of Taiwan (Taipei). They were maintained on a standard diet (Lab diet; PMI Feeds, St. Louis, MO, USA) in the Animal House of National Defense Medical Center and Taipei Veterans General Hospital. Nineteen days after mating, each pregnant mouse was bred in a single cage and was checked twice everyday. Newborn mice received daily vaccination for the first 3 days. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of Taipei Veterans General Hospital and were performed at least twice.

Chemical denaturation. Ovalbumin (OVA; grade V) was obtained from Sigma (St. Louis, MO, USA). Chemical denaturation of OVA was performed as described previously [11]. In brief, it was first denatured by reduction of disulphide bonds after stirring overnight under vacuum suction with 8.0 M urea and 0.2 M 2-mercaptoethanol (Merck, Darmstadt, Germany). Denatured and carboxymethylated OVA (CM-OVA) were prepared by alkylation of the sulphhydryl groups of urea-denatured OVA after stirring for 3 h in the dark with 0.3 M sodium iodoacetate (Sigma) at pH 8.0. The crude preparation of CM-OVA was extensively dialysed against distilled water and then lyophilized.

Adjuvant. A total of 20 base phosphorothioate-modified oligonucleotides with the embedded CpG motifs were synthesized (CpG ODN 1826; Sigma): 5'-TCCAT-GACGTTCCCTGACGTT-3'. Both CpG and CT (Calbiochem, San Diego, CA, USA) were used as adjuvants.

Sublingual vaccination. Each newborn BALB/*c* offspring was sublingually administered with 20 µg OVA or CM-OVA combined with 1 µg CpG or 0.2 µg CT in 1.2 µl phosphate-buffered saline (PBS), once daily for the first 3 days of life, using a 10 µl tip without anaesthesia. Control mice were similarly treated with PBS only.

Systemic sensitization. Mice were sensitized intraperitoneally (i.p.) with 10 µg OVA adsorbed onto 4 mg of alum and suspended in 0.5 ml PBS 7 weeks after vaccination. The mice were bled from the tail and their saliva was collected after the i.p. injection of salivation-stimulating drug, carbamylcholine chloride (10 µg/mouse; Sigma), just before sensitization and 2 weeks after sensitization. The experimental groups and protocol are summarized in Fig. 1A, B respectively.

A Experimental groups

Group	Antigen	Adjuvant	Number
1	OVA	—	6
2	OVA	CT	6
3	OVA	CpG	6
4	CM-OVA	—	6
5	CM-OVA	CT	6
6	CM-OVA	CpG	6
7	PBS		7

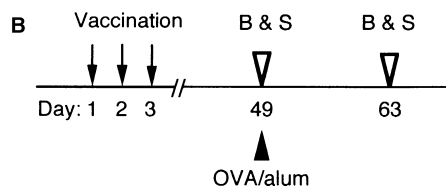


Figure 1 (A) Experimental groups and (B) flowchart. Newborn BALB/*c* offspring were sublingually vaccinated daily for the first 3 days of life. They were sensitized with OVA adsorbed onto alum 7 weeks later. Their blood and saliva were collected just before sensitization and 2 weeks after sensitization. OVA, ovalbumin; CM-OVA, carboxymethylated ovalbumin; CpG, CpG oligodeoxynucleotides; CT, cholera toxin; PBS, phosphate-buffered saline; CLN, cervical lymph node and B & S, blood and saliva sampling.

Assessment of antibody responses by ELISA. Serum anti-OVA antibody and total saliva antibody responses were assessed by enzyme-linked immunosorbent assay (ELISA) as previously described [11]. Briefly, 96-well plates (Nunc, Kamstrup, Roskilde, Denmark) were coated with 100 µg/ml OVA, 10 µg/ml goat anti-mouse IgA (Sigma) or 4 µg/ml affinity-purified rat anti-mouse IgE (Clone 23G3; SBA, Birmingham, AL, USA) in 0.05 M carbonate buffer, pH 9.6 (100 µl/well), overnight at 4 °C. Free sites were blocked with 3% skim milk in PBS-Tween 20 for 1 h. Serum samples (1/8–300), serum standards (pooled hyperimmune sera collected after monthly immunization with 100 µg OVA in Complete Freund's adjuvant for 3 months), saliva sample (1/2–4) and saliva standard (mouse immunoglobulin A; SBA) were added in duplicate and the plates incubated for 5 h at room temperature (RT). After washing, 100 µl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA (1/4000; Sigma), IgG (1/4000; Jackson, West Grove, PA, USA), IgG2a, IgG1 (1/4000 respectively; SBA) or 100 µl of biotinylated OVA (1 µg/ml) were added and the plates incubated for 4 h at RT. For the detection of specific IgE, the plates were washed and streptavidin-HRP (1/3000; Invitrogen, Carlsbad, CA, USA) were added for 1 h at RT. After washing, *o*-phenylenediamine (0.5 mg/ml; Sigma) in citrate-carbonate buffer containing 0.015% H₂O₂ was added and the plates were incubated in the dark. Colour development

was stopped by 4 N sulphuric acid. Absorbance at 492 nm was read using a microplate spectrophotometer (SPECTRAmax 250; Molecular Devices, Sunnyvale, CA, USA) and unknowns were interpolated.

Immunodot assay. Saliva-specific SIgA antibody responses were examined by immunodot. It was performed by applying 100 µg OVA onto a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA, USA). After blocking with 3% skim milk for 2 h, blots were immersed respectively with 1 ml of 1/4 diluted saliva, pooled from six mice of each group overnight at 4 °C. Thereafter, they were immersed in HRP-conjugated goat anti-mouse IgA (1/4000) at RT for 5 h. Blots were washed with Tris-buffered saline after each treatment. At last, blots were immersed in enhanced chemiluminescence reagent (Amersham, Buckinghamshire, UK) for 2 min and exposed to X-ray films (Kodak, Rochester, NY, USA) for 5–30 s at RT. The film was quantified in densitometer (Personal Densitometer™ SI, Amersham Biosciences, Piscataway, NJ, USA).

Cytokine assays. Seven weeks after sublingual treatment, cervical lymph node (CLN) and spleens were collected from four mice in each group. The spleen cells (2×10^6 /well) from each mouse and CLN cells (1×10^6 /well) pooled from the same group were cultured in duplicate in complete RPMI-1640 medium with OVA (1 mg/ml), 10% fetal calf serum and antibiotics in 24-well flat-bottomed microtitre plates (1 ml/well; Costar, Cambridge, MA, USA). Using sandwich ELISA kits (E-bioscience, San Diego, CA, USA), the levels of supernatant interferon (IFN)- γ were detected after 24 h of culture, but the levels of interleukin-4 (IL-4), IL-5 and IL-6 were examined after 72 h of culture.

Statistics. Antibody and cytokine titres were expressed as means + 1SD. Two-tailed Wilcoxon rank sum tests were used for comparison of serum-specific IgG, IgG1, IgG2a and IgE and saliva total SIgA antibody responses and cytokine levels of spleen and CLN cells between groups.

Results

Neonatal sublingual vaccination induces systemic antibody responses before sensitization

The effect of neonatal sublingual vaccination was first studied. The systemic immune responses before sensitization were measured and shown in Fig. 2. Compared with

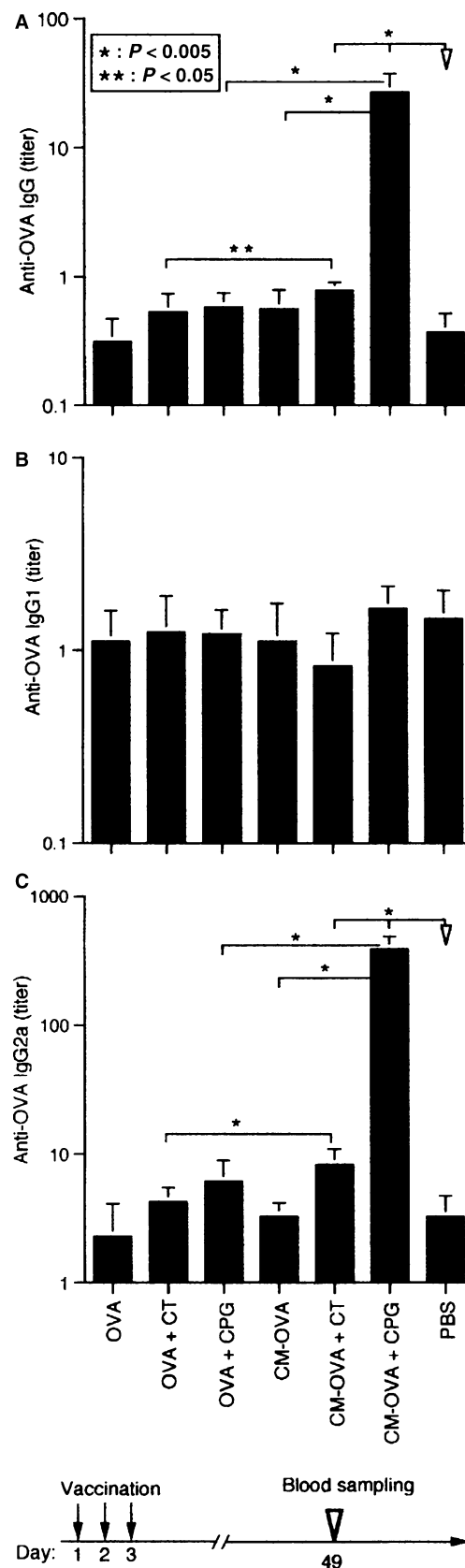


Figure 2 Effect of neonatal sublingual vaccination on systemic antibody responses. Newborn BALB/c mice were sublingually vaccinated daily for the first 3 days with 20 µg OVA or CM-OVA only, or combined with 0.2 µg cholera toxin CT or 1 µg CpG. Seven weeks after the last treatment, their systemic specific antibody responses were examined: (A) IgG, (B) IgG1 and (C) IgG2a. The numbers of the mice in each group and the abbreviations are listed in Fig. 1. * $P < 0.005$, ** $P < 0.05$.

those neonatally receiving PBS or CM-OVA only, the group vaccinated with CM-OVA plus CpG had markedly enhanced specific IgG2a and IgG antibody responses ($P < 0.005$ respectively; Fig. 2). Compared with the mice neonatally receiving OVA combined with CpG, the group receiving CM-OVA plus CpG also had much enhanced specific IgG2a and IgG antibody responses ($P < 0.005$ respectively; Fig. 2). Those neonatally receiving CM-OVA plus CT also had higher specific IgG2a and IgG antibody responses than those receiving PBS ($P < 0.005$ respectively) or those receiving OVA plus CT ($P < 0.005$ and 0.05 respectively; Fig. 2). Specific IgG antibody responses of the mice vaccinated with native OVA were not different from those vaccinated with PBS. In addition, neonatal sublingual vaccination failed to sensitize an IgE antibody response, even in those receiving Th2-dominant adjuvant CT and OVA or CM-OVA (data not shown).

Neonatal sublingual vaccination markedly augments systemic antibody responses after sensitization

The effect of neonatal sublingual vaccination on adult systemic sensitization was further studied and shown in Fig. 3. All the groups neonatally receiving OVA or CM-OVA and adjuvant, CT or CpG, had higher specific IgG antibody responses than the control group ($P < 0.005$ respectively; Fig. 3A) and the group receiving OVA or CM-OVA alone ($P < 0.005$ respectively; Fig. 3A). Whereas the groups receiving adjuvant CT combined with OVA or CM-OVA had much higher IgG1 antibody responses than the controls or those receiving OVA or CM-OVA only ($P < 0.005$ respectively; Fig. 3B), the groups receiving adjuvant CpG together with OVA or CM-OVA had much higher IgG2a antibody responses than the controls or those receiving OVA or CM-OVA only ($P < 0.005$ respectively; Fig. 3C). Again, the group receiving CM-OVA plus CpG or CT had much more enhanced IgG2a antibody responses than those receiving OVA plus CpG or CT ($P < 0.005$ and <0.05 respectively; Fig. 3C). After sensitization, all the groups developed specific IgE antibody responses (Fig. 3D). To compare with the controls, sublingual vaccination with antigen and adjuvant CT did not enhance IgE responses. On the other hand, vaccination with CpG and OVA or CM-OVA, both had significantly suppressed IgE responses ($P < 0.05$ respectively; Fig. 3D).

Neonatal sublingual vaccination effectively induces mucosal antibody responses

The effect of neonatal sublingual vaccination on mucosal SIgA antibody responses was also examined. Either before or after sensitization, the mice neonatally vaccinated with native or denatured OVA plus adjuvant CpG or CT

showed profoundly increased SIgA antibody responses when compared with the other groups (Fig. 4A, B). CT had a stronger adjuvant effect than CpG on specific SIgA production after sensitization (Fig. 4B). Moreover, sublingual vaccination did not affect total saliva SIgA levels (Fig. 4C).

Cytokine secretion of CLN and spleen cells after neonatal sublingual vaccination

The cytokine profiles after neonatal sublingual vaccination were further studied. Compared with the mice neonatally receiving OVA, those receiving CM-OVA showed enhanced cytokine production of IL-5 and IL-6 in spleen cells ($P < 0.05$ respectively; Fig. 5C, D) or IFN- γ and IL-6 in CLN cells (Fig. 6A, D). When compared with the controls, those receiving CM-OVA plus CT had higher levels of IL-4, IL-5 and IL-6 in spleen cells ($P < 0.05$ respectively; Fig. 5) and in CLN cells (Fig. 6). The group neonatally vaccinated with OVA plus CT had higher production of IL-4 in spleen cells ($P < 0.05$; Fig. 5B), or IL-5 and IL-6 in CLN cells (Fig. 6C, D). In contrast, those receiving CpG plus OVA or CM-OVA had significantly enhanced production of IFN- γ in spleen cells ($P < 0.05$ respectively; Fig. 5A) and in CLN cells (Fig. 6A) when compared with the controls. In addition, those receiving CpG or CT together with OVA or CM-OVA had much enhanced IL-6 production in CLN cells (Fig. 6D).

Discussion

This is the first study to demonstrate that neonatal sublingual vaccination with a native or denatured antigen and mucosal adjuvant CT or CpG can preferentially activate systemic and mucosal-specific immune responses in BALB/c mice. Neonatal sublingual vaccination with CM-OVA plus CT can stimulate strong mucosal immunity without suppression of IgE antibody responses. On the contrary, vaccination with CM-OVA and Th1 adjuvant CpG can activate both mucosal and systemic Th1 immunity, including inhibition of IgE antibody production. Consistent with previous reports [12, 13], our data confirm that CpG is also a mucosal adjuvant.

We and other groups have shown that oral administration of a native protein antigen can induce oral tolerance in adult mice, but not in newborns [14–16]. We have further shown here that neonatal sublingual administration of a native protein antigen fails to induce tolerance or priming (Figs. 2, 3). Consistent with the previous reports that both systemic and i.n. immunization with a protein antigen and adjuvant CpG can induce Th1-predominant immunity in adult animals [17, 18], we have shown herein for the first time that neonatal sublingual vaccination with either OVA or CM-OVA

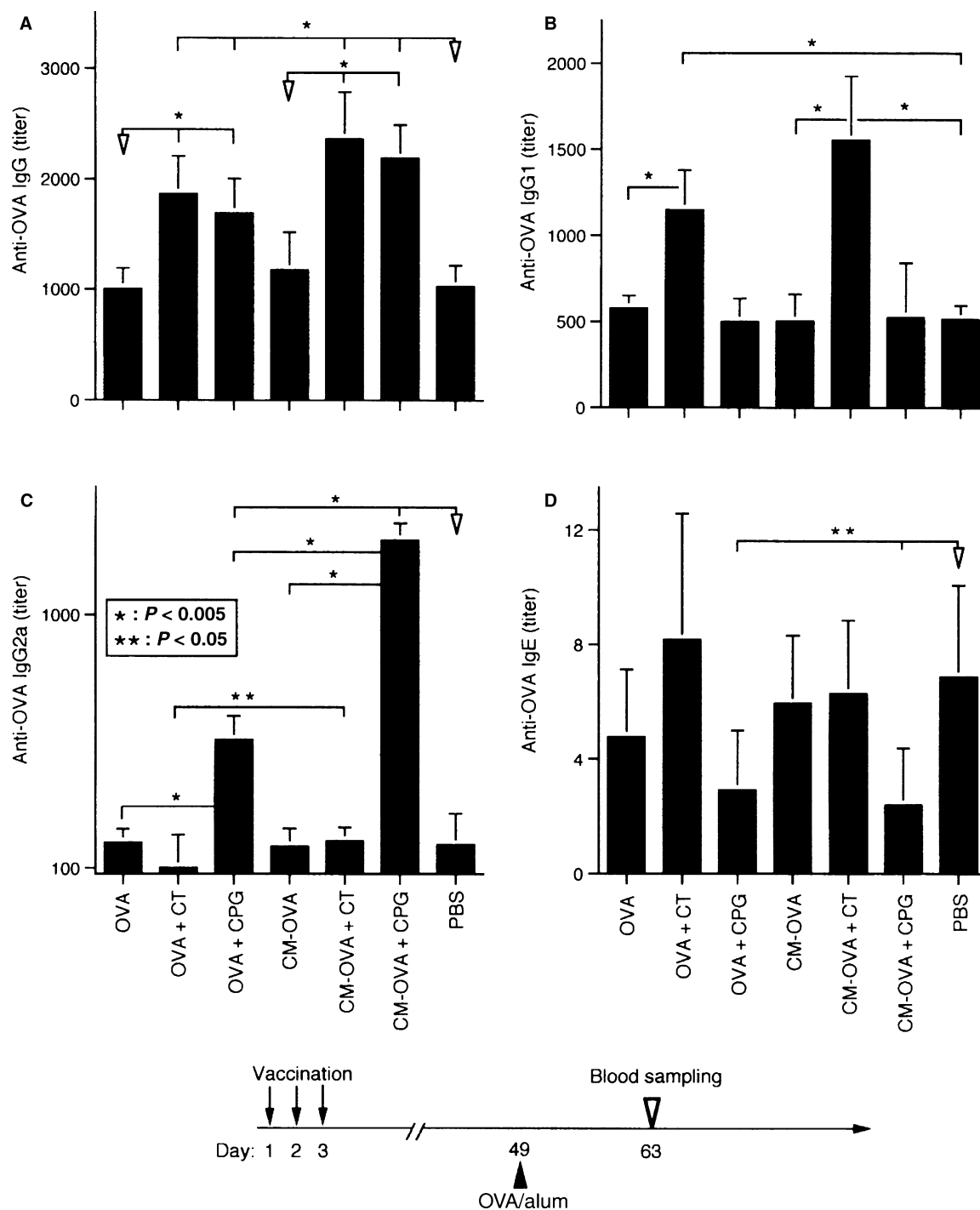


Figure 3 Effect of neonatal sublingual vaccination on systemic antibody responses after sensitization. Newborn BALB/c mice were sublingually vaccinated as described in Fig. 2. Seven weeks after the last vaccination, they were sensitized. Their specific IgG (A), IgG1 (B), IgG2a (C) and IgE (D) antibody responses were examined 2 weeks after sensitization. The numbers of the mice for each group and the abbreviations are listed in Fig. 1.

together with adjuvant CpG also exclusively induces Th1-predominant systemic immune responses. As before [17], they are characterized by serum strong IgG2a antibody responses and by high levels of IFN- γ production in both CLN and spleen cells. On the other hand, adjuvant CT has been shown to predominantly stimulate

Th2-based immune responses [19]. When CT was applied sublingually to the newborns, subsequent sensitization enhanced their IgG1 responses only, but not their IgE responses. Nevertheless, increases of splenic IL-4, IL-5, and IL-6 production in CLN were also found after neonatal sublingual vaccination with CT.

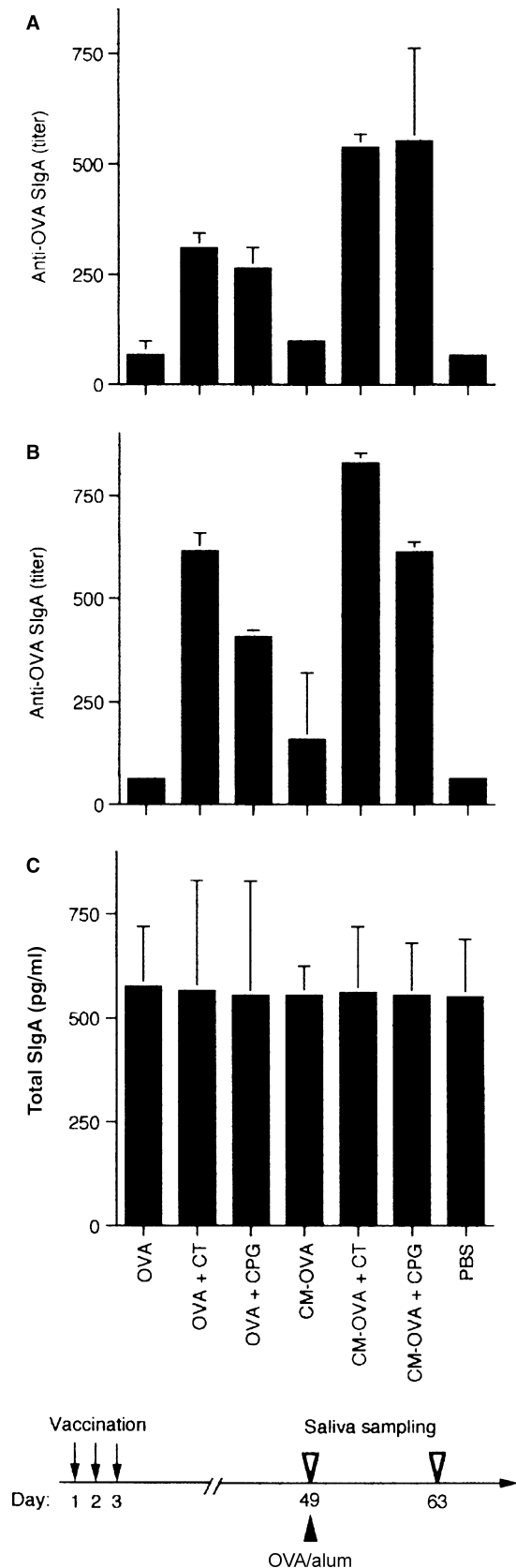


Figure 4 Effect of neonatal sublingual vaccination on mucosal antibody responses. Groups of newborn mice were treated as described in Fig. 2 or 3. The specific saliva secretory IgA (SIgA) antibody response of pooled saliva in each group and the total saliva SIgA level in each mouse were examined respectively and shown as anti-OVA SIgA before sensitization (A), anti-OVA SIgA after sensitization (B) and total SIgA after sensitization (C). The numbers of the mice for each group and the abbreviations are listed in Fig. 1.

Secretory IgA antibodies can act as blocking antibodies to bind luminal antigens and to prevent their penetration through the mucosa. Previous studies have shown that specific mucosal IgA antibodies are crucial to avoid allergen-triggered allergic reactions [20, 21]. This phenomenon has been documented as 'immune exclusion'. Consistent with recent reports that i.n. CT and CpG are potent mucosal adjuvants [18, 19], our study has further shown that neonatal sublingual vaccination together with CT or CpG also can effectively induce specific SIgA antibody responses. Whereas adjuvants, CpG and CT, enhanced both systemic- and mucosal-specific immune responses, they did not affect serum total IgG, IgG1 and IgG2a levels, saliva total SIgA levels and serum circulating cytokines, including IL-4 and IFN- γ (data not shown).

Denaturation of the protein modifies its tertiary structure and leads to random-coiled aggregation and decreased solubility [22, 23]. Thus, its allergenicity can be greatly changed after denaturation [24]. It has been shown that parenteral administration of denatured OVA leads to 10-fold higher frequency of IFN- γ -producing cells than native OVA [25]. It is likely that the particulate form of a protein antigen may be localized in the draining lymph nodes and is thus more effectively processed by antigen-presenting cells; thereby the particulate antigen can induce higher IFN- γ production. This implication is further confirmed by the present study that the newborn mice sublingually receiving denatured OVA plus adjuvant CpG or CT have generated much stronger serum IgG and saliva SIgA antibody responses.

The side effects of injection vaccines include soreness at injection sites and the possibility of lethal anaphylaxis. In addition, injection vaccination is not able to effectively induce mucosal immunity [26]. Therefore, vaccination through mucosal routes is a better choice not only to avoid the side effects of injection but also to induce mucosal immunity. The present study suggests that neonatal sublingual vaccination with antigen and mucosal adjuvant is a rational strategy to reduce the side effects of injection vaccines, but to preferentially enhance specific mucosal and systemic immunity.

The cytokine IL-6 is crucial for the final maturation of IgA⁺ B lymphoblasts into IgA-secreting plasma cells [27]. Indeed, we found here that the IL-6 production of CLN cells in those treated with native or denatured

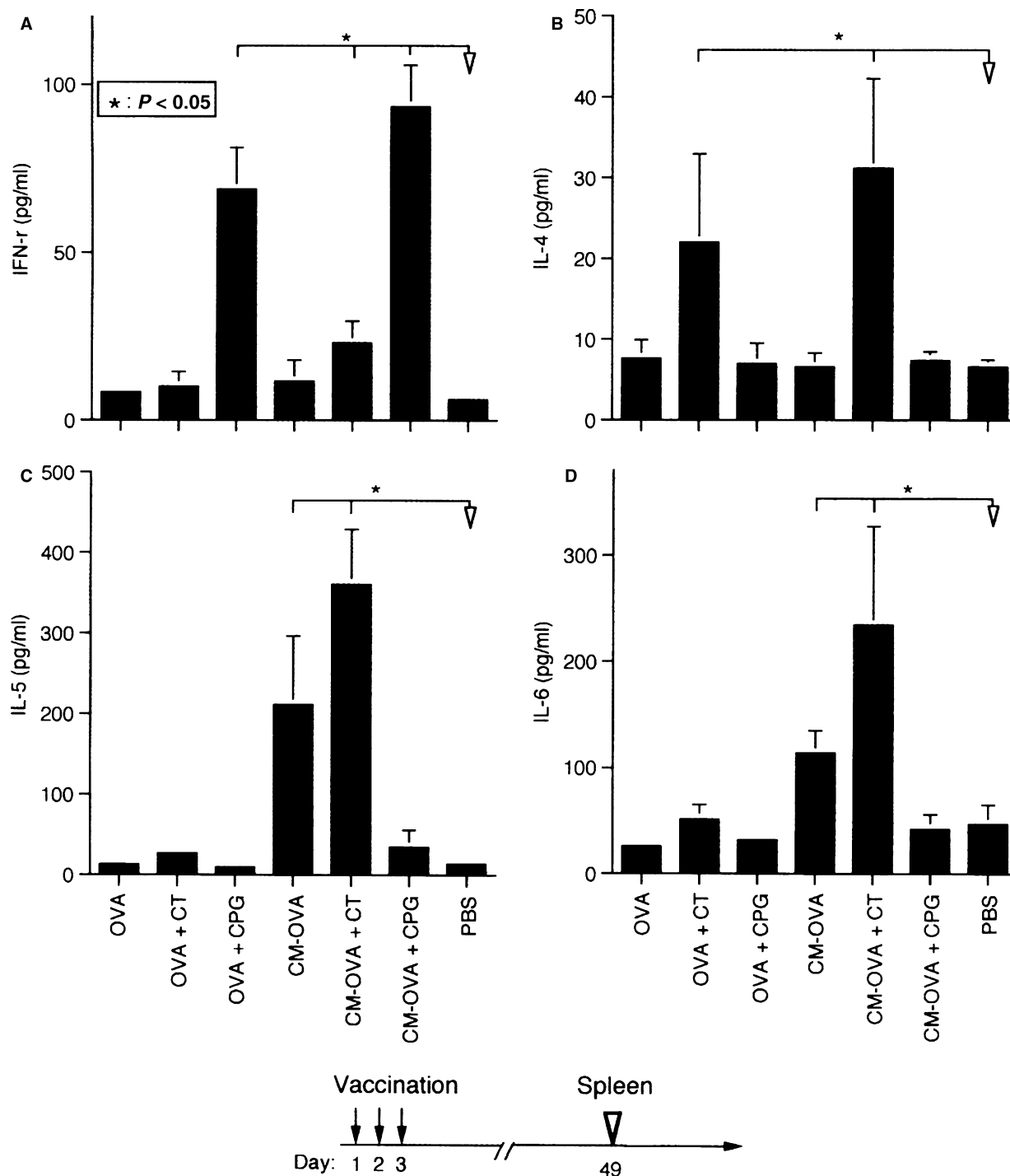


Figure 5 Effect of neonatal sublingual vaccination on cytokine secretion of spleen cells. Four newborn BALB/*c* mice in each group were treated as described in Fig. 2. Seven weeks after the last treatment, their spleens were collected and the spleen cells (2×10^6 /ml) of individual mouse were cultured with OVA (1 mg/ml) *in vitro* for 1 day (interferon γ ; IFN- γ) or 3 days (interleukin-4; IL-4, IL-5 and IL-6) respectively. The cytokines in the supernatant were measured in duplicate using enzyme-linked immunosorbent assay kits and shown as: (A) IFN- γ , (B) IL-4, (C) IL-5 and (D) IL-6. Abbreviations are expanded in the footnote of Fig. 1. * $P < 0.05$.

OVA plus adjuvant CT or CpG was higher than that treated with PBS or OVA alone. Horner *et al.* [28] reported that i.n. co-delivery of β -galactosidase with CpG or CT induced equivalent SIgA immune responses

in adult BALB/*c* mice. Interestingly, our study also revealed that the newborns receiving antigen plus mucosal adjuvant CpG or CT had similarly enhanced SIgA antibody responses. Further investigation using different

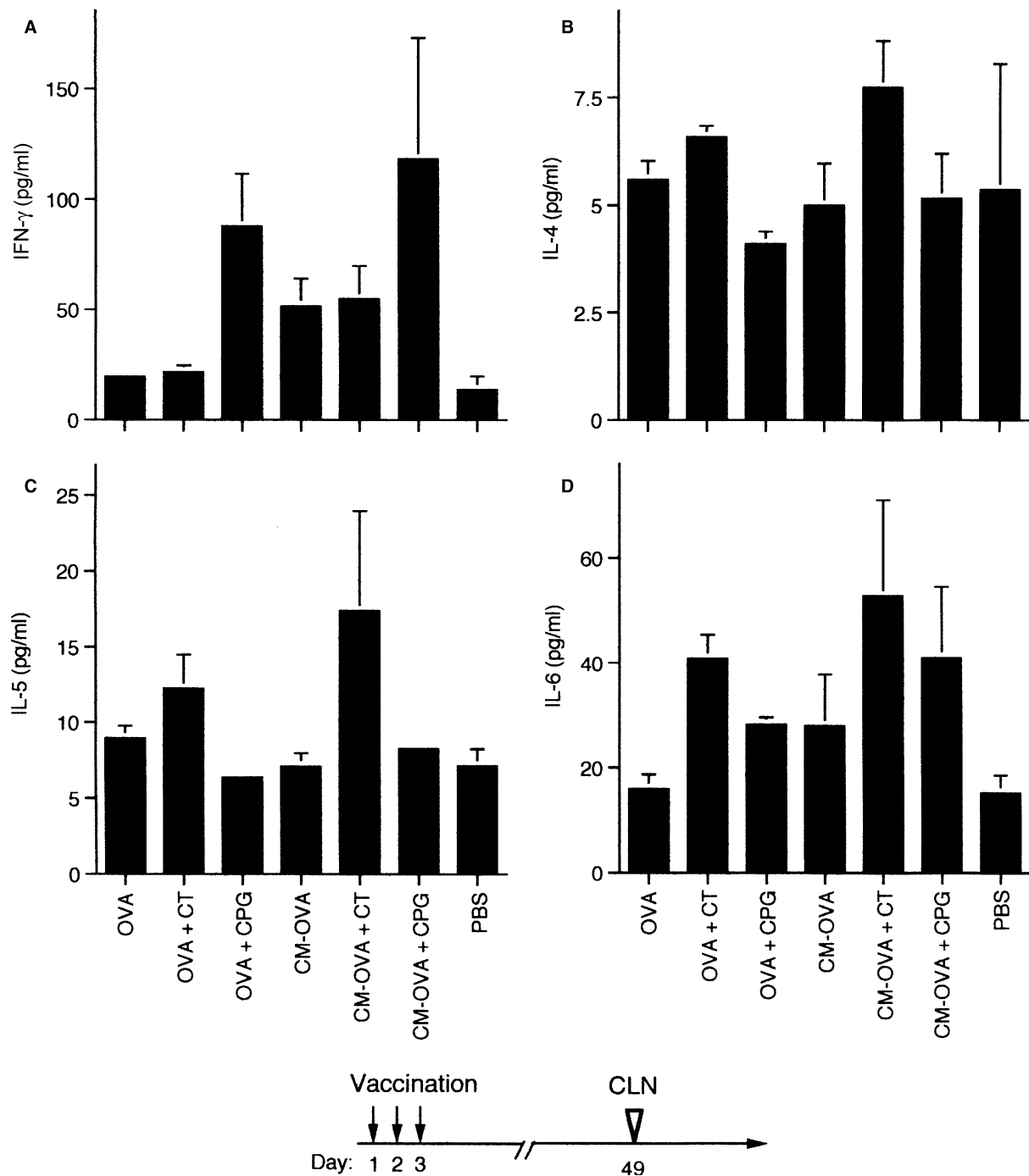


Figure 6 Effect of neonatal sublingual vaccination on cytokine secretion of cervical lymph node (CLN) cells. Four newborn BALB/c mice in each group were treated as described in Fig. 5. Seven weeks after the last treatment, their CLNs were collected and CLN cells from the same group were mixed and (1×10^6 /ml) cultured with OVA (1 mg/ml) for 1 or 3 days respectively. The cytokines in the supernatant were measured in duplicate using enzyme-linked immunosorbent assay kits and shown as: (A) interferon γ (IFN- γ), (B) interleukin-4 (IL-4), (C) IL-5 and (D) IL-6. These animal experiments were repeated for three times. Abbreviations are expanded in the footnote of Fig. 1.

dosage of CpG and CT is still needed to further elucidate their discrepancy.

Our recent studies in adult mice have shown that sublingual vaccination with denatured OVA plus adjuvant

CpG can effectively induce specific mucosal and systemic Th1 responses and prevent the subsequent induction of IgE reactions simultaneously [29]. In agreement with the studies in adult mice, the present studies have further

shown that neonatal sublingual vaccination with a denatured allergen and mucosal adjuvant CpG can also effectively induce mucosal and systemic immune responses and prevent allergic sensitization. Furthermore, neonatal sublingual vaccination with aeroallergens or bacterial antigens might confer an immunoprophylactic effect and needs extensive investigation.

Acknowledgment

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