

## Intranasal application of purified protein derivative suppresses the initiation but not the exacerbation of allergic rhinitis in mice

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### Summary

**Background** Several epidemiological and experimental studies have demonstrated that exposure to pathogens such as those from the genus *Mycobacterium* leads to the suppression of allergic sensitization and inflammation. However, little is known as to whether pathogen-derived soluble antigens have the potential to modulate the pathogenesis of allergic rhinitis.

**Objective** We sought to determine whether application of purified protein derivative (PPD) from *Mycobacterium tuberculosis* can suppress the initiation and/or exacerbation of allergic rhinitis using a recently developed murine model.

**Methods** First, we investigated whether a single intranasal application of PPD could elicit cytokine production in the nose by RT-PCR. BALB/c mice were repeatedly sensitized with *Schistosoma mansoni* egg antigen (SEA) intranasally without an adjuvant. PPD was applied through different routes either before or after sensitization. The production of SEA-specific antibodies, nasal eosinophilia and cytokines by nasal lymphocytes was compared among mice that had or had not received PPD treatment.

**Results** IFN- $\gamma$ , but not IL-4, was detected in the nasal tissue 12 to 48 h after a single intranasal application of 10  $\mu$ g PPD. Repeated intranasal application of PPD prior to and during sensitization with SEA significantly inhibited the production of both SEA-specific IgE/IgG1 and nasal eosinophilia. Moreover, it partially inhibited the production of IL-4 by nasal lymphocytes in response to SEA. Conversely, this treatment led to a significant increase in IFN- $\gamma$  production. On the other hand, PPD applied through the footpad had no effect over the same period. Repeated intranasal application of PPD after sensitization with SEA had no exacerbative effect on allergic inflammation.

**Conclusion** These results indicate that the local application of PPD, and the subsequent induction of IFN- $\gamma$ , inhibits the initiation, but not the exacerbation, of allergic rhinitis in mice. This suggests that pathogen-derived antigens have potential for use in the prevention and prophylaxis of allergic rhinitis.

**Keywords** allergic rhinitis, eosinophil, IFN- $\gamma$ , IgE, IL-4, mice, mycobacterium, nose, PPD

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### Introduction

Allergic rhinitis is the most common allergic disease and is characterized by the production of allergen-specific IgE and nasal eosinophilia [1]. In developed countries, more than 10% of the population suffer from allergic rhinitis and the disease creates burdens such as medical expenses and loss of productivity [2]. Previous studies have indicated that Th2-type cytokines, such as IL-4 and IL-5, play a pivotal role in the pathogenesis of the disease [3]. It is widely accepted that IL-4 induces the differentiation and expansion of Th2 lymphocytes, which in turn produce IL-4, IL-5, IL-6, IL-10 and IL-13 [4, 5]. IL-4 also

plays a critical role in the production of IgE, which mediates immediate hypersensitivity reactions [6, 7]. Furthermore, IL-4 up-regulates the expression of VCAM-1 on vascular endothelial cells. VCAM-1 is a ligand for VLA-4, which is expressed on eosinophils and basophils but is absent on neutrophils [8]. IL-5 is particularly important in allergic inflammation as it selectively promotes chemotaxis, activation and the survival of eosinophils [9–11].

One strategy to prevent the initiation and/or exacerbation of allergic disease involves skewing local inflammatory sites (i.e. the nasal mucosa of subjects with allergic rhinitis) towards a Th1-dominated micro-environment, as Th1-associated cytokines down-regulate Th2 function [6]. In fact, several epidemiological studies support this hypothesis [12–14]. For example, the prevalence of allergic rhinitis in subjects with a history of viral hepatitis, which causes Th1 immune responses, is significantly lower than that of other segments of

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the population [13]. In addition, an inverse relationship has been observed between the total amount of serum IgE measured and the degree of delayed-type hypersensitivity (DHT) following intradermal injection with purified protein derivative of *Mycobacterium tuberculosis* (PPD), in Japan [14]. Furthermore, studies using experimental animals have shown that when a Th1-dominated immune environment is established by prior infection or vaccination with live, attenuated or killed pathogen, Th2 allergic inflammation is suppressed [15–17]. Of the pathogens studied, those from the genus *Mycobacterium* appear to be the most potent inducers of Th1-type responses [18].

We recently developed a murine model of allergic rhinitis [19]. Using this model, the repeated intranasal administration of *Shistosoma mansoni* egg antigen (SEA), in the absence of an adjuvant, induced a Th2 response and led to the production of SEA-specific IgE as well as eosinophil infiltration into the nasal mucosa [19]. We used this model to determine whether pathogen-derived antigen can solely suppress the initiation and/or exacerbation of allergic rhinitis, in the present study. We chose PPD, rather than Bacillus Calmette-Guerin (BCG), as the Th1-inducer as the *in vivo* application of BCG is known to induce granuloma formation in the lung and liver [16, 17] and thus, may be harmful for clinical use. Our results are the first to demonstrate that the local application of PPD induces IFN- $\gamma$  in the nasal mucosa and suppresses the initiation of allergic rhinitis. These results provide evidence with regard to the role of pathogens and pathogen-derived antigens in altering inflammation in allergic rhinitis. Moreover, they suggest the potential merits of a new approach for regulating allergic rhinitis via the intranasal application of Th1-inducible antigens during the critical period of sensitization.

## Materials and methods

### Animals

Female BALB/c mice aged 7 to 10 weeks old were purchased from Charles River Japan (Yokohama, Japan). The mice were maintained in SPF conditions at Okayama University Medical School in accordance with the guidelines set forth by the Okayama University Medical Area Research Committee.

### Antigens

A Puerto Rican strain of *Schistosoma mansoni* was maintained using Biomphalaria glabrata snails and CD-1 mice (Taconic Farms, Germantown, PA, USA). SEA was prepared from eggs harvested from the livers of CD-1 mice 7 to 8 weeks after intraperitoneal infection with 200 cercaria, as previously described [20]. PPD was purchased from Nihon BCG Seizo Co. (Tokyo, Japan).

### Determination of cytokine signals after intranasal application of antigens

To determine this, 0.1, 1 or 10  $\mu$ g of PPD, or 5  $\mu$ g of SEA dissolved in 20  $\mu$ L PBS ( $n = 4$ /group), were intranasally applied to BALB/c mice. Before and 12, 24, 48 and 72 h after application, mRNA was extracted from the nasal tissue, then IL-4, IFN- $\gamma$  and  $\beta$ -actin were detected by RT-PCR, as described elsewhere [21].

### Sensitization of mice

We designed two procedures for intranasal sensitization in order to investigate the effects of PPD application on either the initiation or exacerbation of allergic rhinitis in our recently developed murine model [19]. In order to determine whether PPD application can alter the initiation of allergic rhinitis, 10  $\mu$ g of PPD in 20  $\mu$ L PBS was applied intranasally once a week for 3 weeks, then 5  $\mu$ g of SEA mixed with 10  $\mu$ g of PPD was inoculated into the nostril once a week for 3 weeks. One week later, 1  $\mu$ g of SEA mixed with 2  $\mu$ g of PPD was challenged intranasally for 7 consecutive days (Fig. 1a). Blood was sampled from the tail 1 day prior to sensitization with SEA, 5 days after the third sensitization with SEA, and 12 h after the final nasal challenge. In addition, 12 h after the final challenge, the mice were sacrificed and nasal sections were obtained (Fig. 1a). In another set of experiments, an equal amount of PPD to that used for intranasal application was administered through the footpad in order to determine whether the route of PPD application makes a difference in altering SEA sensitization.

Next, we sought to determine whether intranasal application of PPD can alter the exacerbation of allergic inflammation in intranasally pre-sensitized mice. First, mice were sensitized intranasally with 5  $\mu$ g of SEA once a week for 3 weeks. Sensitization to SEA was confirmed by the presence of SEA-specific IgE 5 days after the third sensitization. Then we applied 10  $\mu$ g of PPD intranasally one, three or five times, over 2 weeks. Following the 2-week PPD treatment, mice were challenged

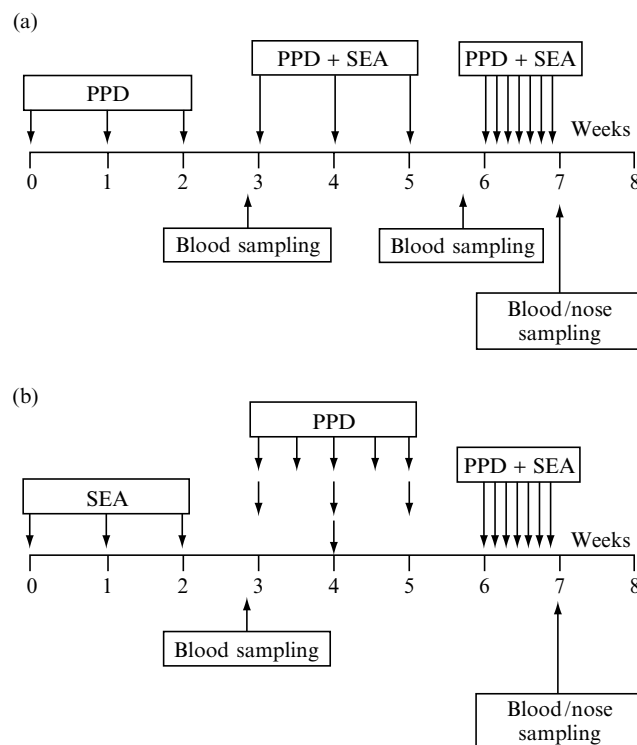


**Fig. 1.** Experimental design used to investigate the effect of PPD application on the initiation (a) or exacerbation (b) of allergic rhinitis in mice. BALB/c mice were sensitized and subsequently challenged with SEA in the absence of an adjuvant. PPD was given intranasally or injected into the footpad prior to and during sensitization with SEA (a), or after sensitization (b). Sensitization was confirmed by the presence of SEA-specific IgE.

with 1 µg of SEA mixed with 2 µg of PPD for 7 consecutive days, and blood samples and nasal sections were obtained 12 h after the final challenge (Fig. 1b).

#### Serum Ab titres

Antigen-specific IgE was determined by sandwich ELISA using biotinylated antigen as the detection reagent, as previously described [7, 19]. Titres are expressed as the mean OD at 450 nm of six individual serum samples diluted to 1:4. Ag-specific IgG1 and IgG2a Ab were detected by indirect ELISA, as previously described [7, 19]. Titres are expressed as the mean OD at 450 nm of six individual serum samples diluted to 1:100.

#### Histological examination

Histological examination was performed as previously described with a slight modification [7, 19]. In brief, 12 h after the final nasal challenge, mice were killed by cervical dislocation. Their heads were removed, fixed in 10% formalin and decalcified with 2.5% EDTA-2Na solution. Coronal nasal sections were stained with Luna solution and the number of eosinophils in the nasal mucosa was counted microscopically under a high power field ( $10 \times 40$ ).

#### In vitro culture of nasal lymphocytes

Twelve hours following the final nasal challenge, mice were sacrificed and their nasal lymphocytes were isolated by enzyme extraction using collagenase, as described by Asanuma *et al.* [22]. Viable lymphocytes were counted by trypan blue exclusion with a Neubauer haemocytometer. Cell suspensions were adjusted to  $2 \times 10^6$  cells/mL in RPMI 1640 medium (Gibco, NY, USA) containing 10% FCS, and 100 units/mL: 100 µg/mL of penicillin: streptomycin (Sigma). Cells were cultured in flat bottomed 48-well plates (Corning) with SEA (5 µg/mL) and either ConA (2 µg/mL), which served as a positive control, or supplemented medium, which served as a negative control. Following incubation at 37 °C for 72 h in 5% CO<sub>2</sub>, supernatant was collected and stored at -80 °C until assayed for cytokine production.

#### Cytokine determination

IL-4 and IFN-γ production by stimulated (SEA and ConA) and unstimulated nasal lymphocytes was measured by sandwich ELISA, as previously described [23]. Detection limits for IL-4 and IFN-γ were 0.1 U/mL and 1.0 U/mL, respectively.

#### Statistical analysis

Student's unpaired *t*-test was used to determine the statistical significance of the values obtained.  $P < 0.05$  was considered statistically significant. Data are expressed as the mean  $\pm$  standard error of mean (SEM) for each subject group.

## Results

#### Effect of intranasal application of PPD on the induction of cytokines

A total of 10 µg of PPD was intranasally applied to BALB/c mice and mRNA was subsequently extracted from the nasal tissues. Then IL-4, IFN-γ and β-actin were detected by RT-PCR. IL-4 was not detected during the period of study;

however, INF-γ could be detected 12 to 48 h following a single application of 10 µg PPD (Fig. 2a). On the other hand, IL-4 was detected within the nasal tissues 12 to 28 h following the intranasal application of 5 µg SEA (Fig. 1b). In addition, 1 µg or less of PPD did not induce nasal production of IFN-γ (data not shown). These results indicate that 10 µg of PPD appears to induce a Th1-dominated micro-environment in BALB/c noses; thus we have chosen this amount for *in vivo* application in our further investigations.

#### Effect of intranasal application of PPD on the initiation of allergic rhinitis

We sought to determine the effect of PPD on the initiation of allergic rhinitis in a murine model we have recently developed [7]. As described in Materials and methods, the intranasal application of PPD was performed prior to and during sensitization with SEA (Fig. 1a). The production of SEA-specific IgE was significantly lower in mice treated with PPD, compared with mice that did not receive PPD treatment (Fig. 3a). In addition, the production of specific IgG1 was also lower in mice treated with PPD (Fig. 3b). However, the production of specific IgG2a was inversely increased in these mice, although the difference observed was not significant (Fig. 3c).

Following nasal challenge with SEA, eosinophil infiltration into the nasal septum was significantly lower ( $P < 0.01$ ) in mice treated with PPD, compared with those that did not receive PPD treatment (Fig. 4). On the other hand, no histological changes were seen in the lungs of either PPD-treated or untreated SEA-sensitized mice (data not shown). The average number  $\pm$  SEM of eosinophils infiltrating into the nasal septum per field ( $10 \times 40$ ) was  $79.83 \pm 9.06$  in mice sensitized with SEA alone and  $43.17 \pm 9.13$  in mice treated with PPD.

After the final nasal challenge, nasal lymphocytes were isolated and stimulated with ConA or SEA for 72 h, and the culture supernatant was assayed for cytokine production. SEA-stimulated nasal lymphocytes from PPD untreated mice produced Th2-associated IL-4 but not Th1-associated IFN-γ

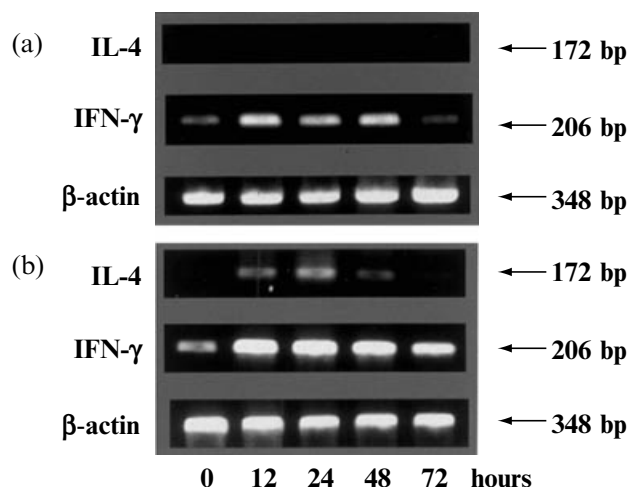


**Fig. 2.** Expression of IL-4, IFN-γ and β-actin in the nasal tissues after a single intranasal application of PPD (a) or SEA (b). 10 µg PPD (a) or 5 µg SEA (b) in 20 µL PBS was applied intranasally through the nostril in BALB/c mice ( $n = 4$ ). Before and 12, 24, 48 and 72 h after application, mRNA was extracted from the nose, then the levels of IL-4, IFN-γ and β-actin were detected by RT-PCR, as described in Materials and methods.

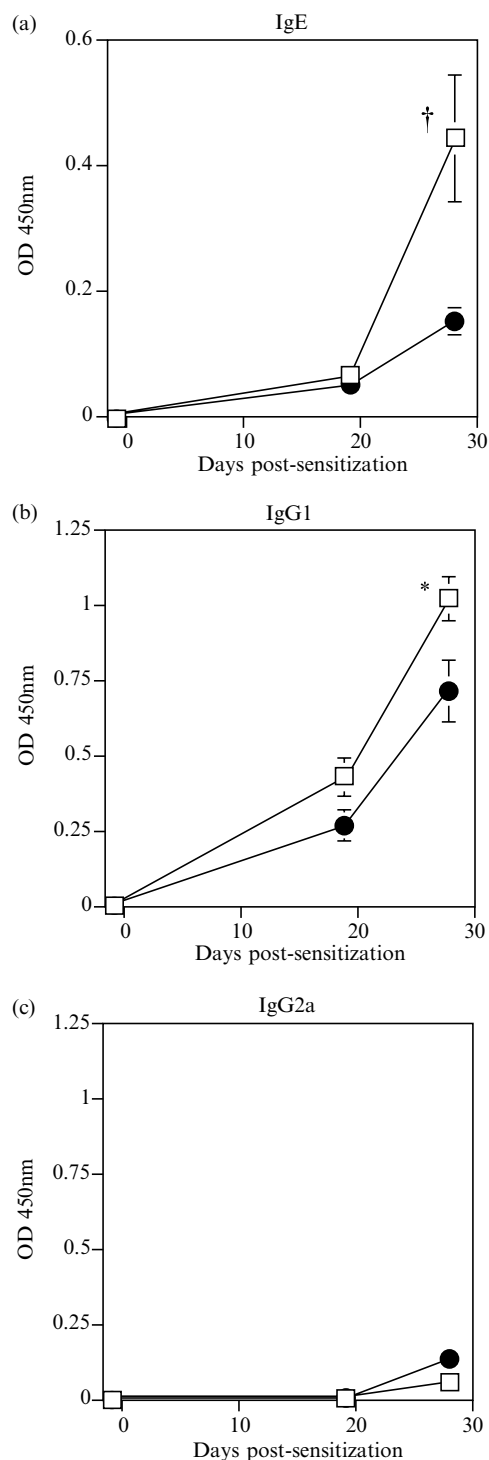


**Fig. 3.** Effect of intranasal application of PPD on the induction of antibody production. BALB/c mice ( $n = 6$  per group) were intranasally sensitized with SEA. PPD (●) or PBS (□), which served as a control, was applied intranasally prior to and during sensitization and subsequent challenge, as shown in Fig. 1(a). Blood was sampled from the tail 1 day prior to sensitization with SEA, 5 days after the third sensitization with SEA, and 12 h after the final nasal challenge. Titres of SEA-specific IgE (a), IgG1 (b) and IgG2a (c) were determined by ELISA. Results show (a) the mean OD at 450 nm  $\pm$  SEM of six serum samples from each group at a 1:4 dilution, and (b, c) the mean OD at 450 nm  $\pm$  SEM of six serum samples from each group at a 1:100 dilution. Data are representative of three separate experiments. \* $P < 0.05$ ; † $P < 0.01$ .

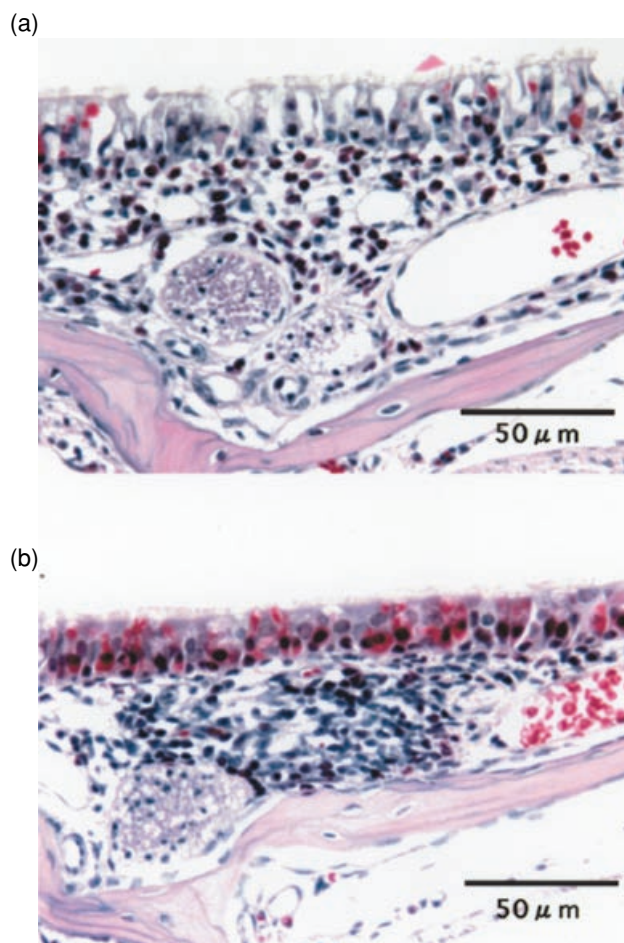


**Fig. 4.** Effect of intranasal application of PPD on the induction of nasal eosinophilia. BALB/c mice ( $n = 6$  per group) were intranasally sensitized with SEA. PBS (a) or PPD (b) was applied intranasally prior to and during sensitization and subsequent challenge. 12 h after the final nasal challenge with SEA, mice were sacrificed. Nasal sections were fixed, decalcified, and Luna staining was performed to detect eosinophils in the nasal mucosa.

(Fig. 5). Nasal lymphocytes from mice intranasally treated with PPD showed an increased production of IFN- $\gamma$  in response to SEA. In addition, the production of IL-4 in response to SEA was reduced in mice treated with PPD, albeit the difference observed was not significant ( $P < 0.1$ ). Similar results were also seen when cells were stimulated with ConA. These results indicate that the intranasal application of PPD prior to and during sensitization with SEA can suppress the initiation of allergic rhinitis in BALB/c mice.

#### *Effect of footpad application of PPD on the initiation of allergic rhinitis*

In order to determine whether the suppressive effect of PPD on the initiation of allergic rhinitis is dependent on the route of application, we injected mice with 10  $\mu$ g of PPD via the footpad, following the same time line used to study the effects of intranasal application. Unlike that observed for mice that were intranasally inoculated with PPD, the footpad application of PPD had no effect on the initiation of allergic rhinitis. Mice that received repeated PPD applications into their footpad produced SEA-specific IgE and IgG1, the amounts of which were

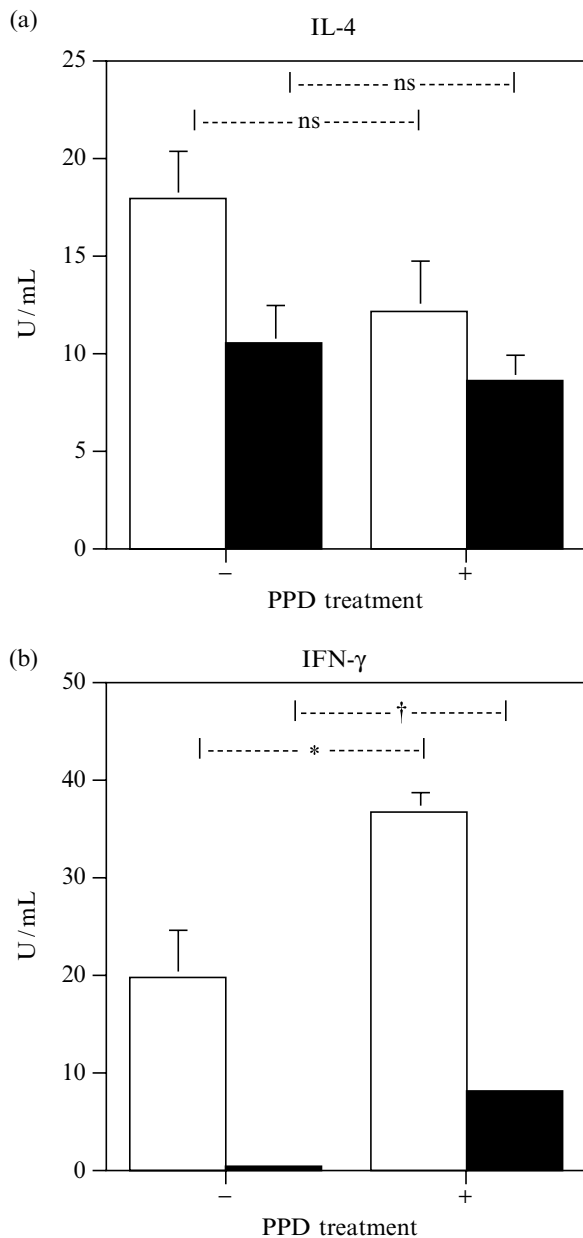


**Fig. 5.** Production of IL-4 (a) and IFN- $\gamma$  (b) by nasal lymphocytes from BALB/c mice treated with PPD or PBS intranasally prior to and during sensitization and subsequent challenge with SEA. Nasal lymphocytes were isolated and cultured *in vitro* for 72 h with ConA (□) or SEA (■). Cytokines were measured by ELISA. Results show the mean  $\pm$  SEM of three separate experiments. \* $P < 0.05$ ; † $P < 0.01$ .

comparable with that produced by mice that had not received PPD treatment (Fig. 6). In addition, following nasal challenge with SEA, eosinophil infiltration into the nasal septum was similar among mice that had and had not received PPD treatment via the footpad (data not shown).

#### *Effect of intranasal application of PPD on the ongoing exacerbation of allergic rhinitis in intranasally pre-sensitized mice*

Next, we investigated whether intranasal application of PPD is also effective at suppressing ongoing allergic inflammation in

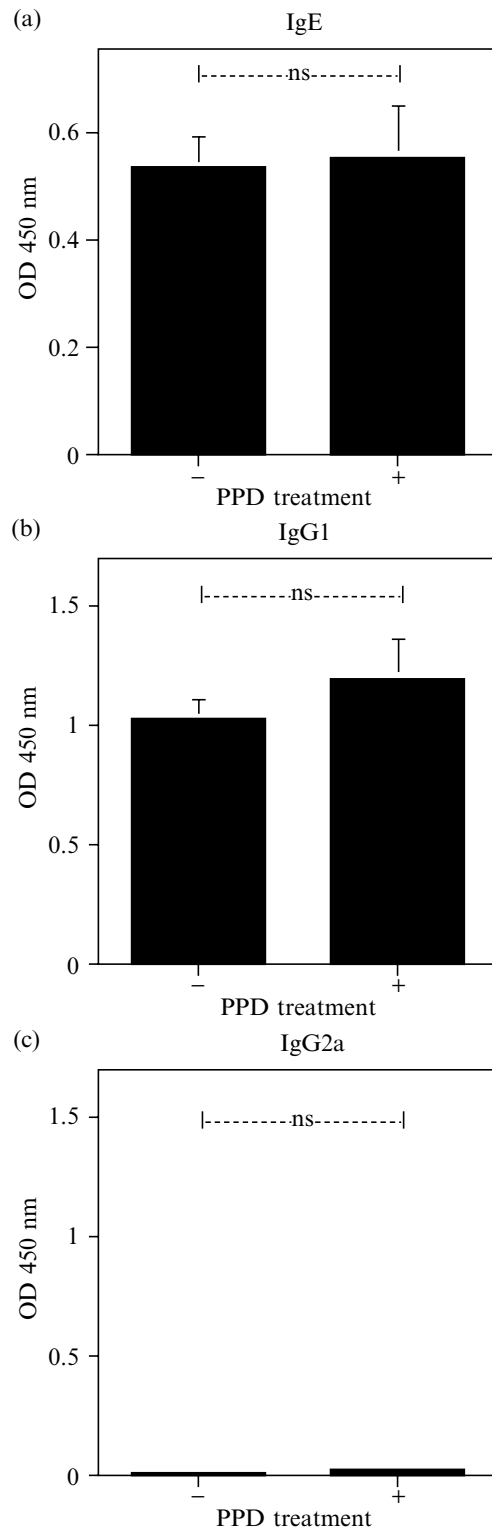


**Fig. 6.** Effect of footpad application of PPD on the induction of antibody production. BALB/c mice ( $n = 6$  per group) were intranasally sensitized with SEA. PPD, or PBS as a control, was delivered into the footpad prior to and during sensitization and subsequent challenge. Blood was sampled from the tail 12 h after the final nasal challenge with SEA. Titres of SEA-specific IgE (a), IgG1 (b) and IgG2a (c) were determined by ELISA. Results show (a) the mean OD at 450 nm  $\pm$  SEM of six serum samples from each group at a 1:4 dilution, and (b, c) the mean OD at 450 nm  $\pm$  SEM of six serum samples from each group at a 1:100 dilution. Data are representative of two separate experiments.

intranasally pre-sensitized mice (Fig. 1b). In this experiment, mice treated with PPD produced increased amounts of specific IgE and IgG1, similar to that produced by PPD untreated mice (Fig. 7). In addition, similar numbers of nasal eosinophils were seen in both PPD-treated and untreated mice (Fig. 8). The average number  $\pm$  SEM of eosinophils infiltrating the nasal septum per field ( $10 \times 40$ ) after intranasal sensitization with SEA was  $124.67 \pm 17.17$ ,  $106.50 \pm 17.17$  and  $108.67 \pm 10.30$  in mice treated with PPD intranasally one, two and three times, respectively;  $131.67 \pm 16.20$  eosinophils per field were detected in the nasal septum of PPD untreated mice. These results suggest that the intranasal application of PPD does not have a suppressive effect on the exacerbation of allergic inflammation in intranasally pre-sensitized mice.

## Discussion

In the present study, we used PPD as a potential moderator of the initiation and exacerbation of allergic rhinitis as this antigen is known to induce strong IFN- $\gamma$  production in both humans and mice [24, 25]. However, little is known regarding the effect of PPD on the nasal mucosa, the actual inflammatory site of allergic rhinitis. We first demonstrated that a single application of  $10 \mu\text{g}$  PPD into the nostril induces the signals for IFN- $\gamma$  in the nose. IFN- $\gamma$  was detected 12 to 48 h after application. However, IL-4 was not detected throughout the period of observation. Our results are consistent with a report in which the intravenous embolization of polymer beads bound with PPD into the lungs of BALB/c mice induced INF- $\gamma$  but not IL-4 production by lung granulomas on day 2 [25]. Moreover, no significant histological changes, such as granuloma formation, were observed in the lungs of mice involved in our procedure. These results encouraged us to argue the potential merits of intranasal application of PPD for the suppression of Th2 allergic inflammation of the nose because the application of PPD appears to be harmless, in comparison with BCG, in terms of granuloma formation [16, 17].

Mice that received PPD intranasally prior to and during intranasal sensitization with SEA, produced significantly less SEA-specific IgE and IgG1 Abs, which are positively regulated by Th2 cytokines, especially IL-4 [6]. In addition, this treatment increased the production of Th1-associated IgG2a, although the difference observed was not significant. Herz et al. demonstrated that intravenous BCG infection, prior to systemic OVA sensitization, suppresses the production of OVA-specific IgE and IgG1 Abs and doubles the production of anti-OVA IgG2a Ab [17]. Scott et al. reported that the concomitant application of heat-killed *Brucella abortus* (BA) coupled with sensitization with OVA adsorbed to alum, suppresses the high IgE response [15]. On the other hand, Erb et al. reported that intranasal BCG infection before systemic OVA sensitization does not affect the production of OVA-specific Abs [16]. Our results are consistent with the former reports and suggest that the intranasal application of a stimulus capable of inducing IFN- $\gamma$  production can inhibit the synthesis of Th2-type Abs after local sensitization without an adjuvant. In fact, local isotype switching to IgE following nasal challenge was observed in the nasal mucosa by the detection of clones of deleted switch circular DNA (Sepsilon/Smu), representing a mu to epsilon switch in nasal lavage cells [26].

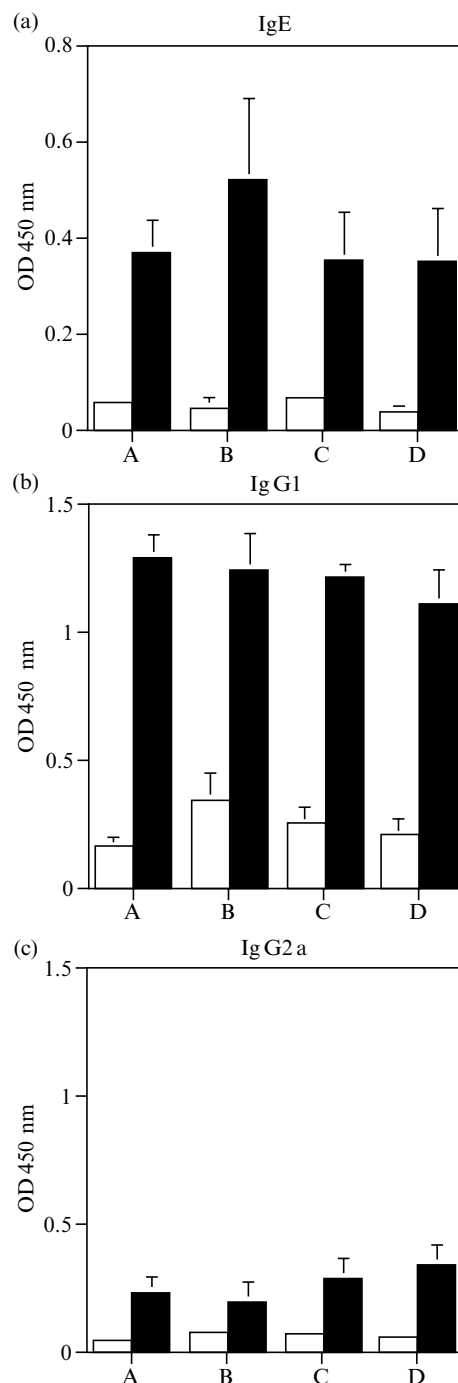


**Fig. 7.** Effect of intranasal application of PPD on the exacerbation of antibody production. BALB/c mice ( $n = 6$  per group) were intranasally sensitized with SEA. Serum was withdrawn 5 days after the third sensitization, at which point we could detect similar amounts of SEA-specific IgE, IgG1 and IgG2a among the groups ( $\square$ ). Then  $10 \mu\text{g}$  of PPD was applied intranasally one (B), three (C) or five times (D) over 2 weeks, as shown in Fig. 1(b). After the 2-week PPD treatment, mice were challenged with  $1 \mu\text{g}$  of SEA mixed with  $2 \mu\text{g}$  of PPD for 7 consecutive days, and blood samples were obtained 12 h after the final challenge ( $\blacksquare$ ). Control mice were given SEA alone throughout the study (A). Titres of SEA-specific IgE (a), IgG1 (b) and IgG2a (c) were determined by ELISA. Results show (a) the mean OD at 450 nm  $\pm$  SEM of six serum samples from each group at a 1:4 dilution, and (b, c) the mean OD at 450 nm  $\pm$  SEM of six serum samples from each group at a 1:100 dilution. Data are representative of two separate experiments.

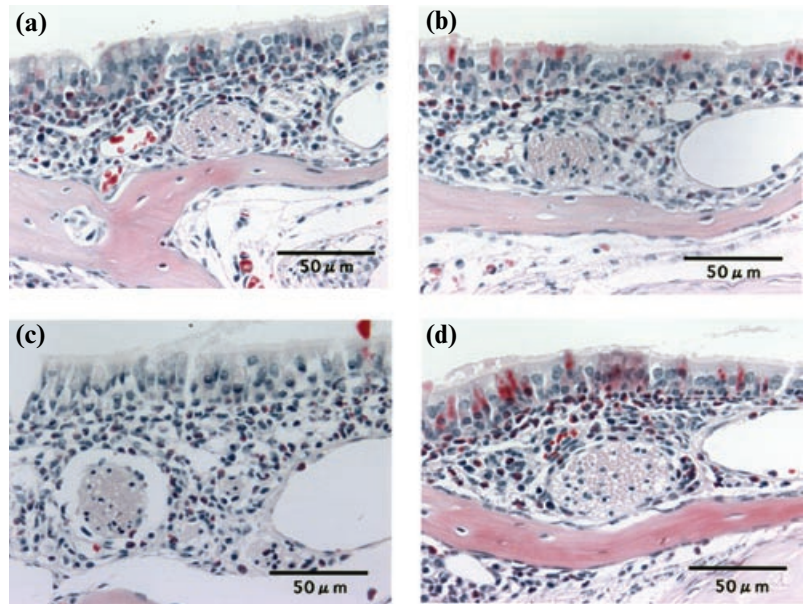


**Fig. 8.** Effect of intranasal application of PPD on the exacerbation of nasal eosinophilia. BALB/c mice ( $n = 6$  per group) were intranasally sensitized with SEA. Then 10 mg of PPD was applied intranasally one (b), three (c) or five times (d) over 2 weeks. After the 2-week PPD treatment, mice were challenged with 1 µg of SEA mixed with 2 µg of PPD for 7 consecutive days, and their heads were removed 12 h after the final challenge. Control mice were given SEA alone throughout the study (a). Nasal sections were fixed, decalcified, and Luna staining was performed to detect eosinophils in the nasal mucosa.

Mice that received PPD intranasally prior to and during intranasal sensitization with SEA, further showed a significant reduction in eosinophil infiltration into the nasal mucosa after intranasal challenge with SEA. Previous reports have demonstrated that BCG infection prior to systemic sensitization with OVA suppresses the accumulation of eosinophils in BAL fluid after local challenge with OVA [16, 17]. Our results confirm this and suggest that, not only can the mycobacterium itself, but also a soluble protein antigen from the pathogen, elicit a suppressive effect on local eosinophilia when inoculated locally and prior to sensitization with allergens.

In addition, the nasal lymphocytes from mice that received PPD treatment prior to and during sensitization with SEA produced lesser amounts of IL-4 and significantly greater amounts of IFN- $\gamma$  in response to SEA or ConA, compared with those that did not receive PPD treatment. Hertz et al. reported that splenic mononuclear cells from mice that were injected intravenously with BCG prior to intraperitoneal sensitization with OVA produced significantly lower amounts of IL-4 and greater amounts of IFN- $\gamma$  in response to ConA, compared with those that did not receive BCG treatment [17]. Yang et al. also showed that OVA-driven IFN- $\gamma$  production is significantly greater in BCG-infected mice compared with those not infected [27]. Summarizing these results, local PPD application prior to and during intranasal sensitization with SEA inhibits initiation of the allergic Th2 response by skewing the micro-environment towards Th1.

Importantly, the suppressive effect of PPD on the initiation of allergic rhinitis depends on the route of application. Intranasal application of PPD was observed to suppress the initiation of allergic rhinitis, whereas application of antigen into the footpad had no such effect. There is a debate with regard to whether BCG vaccination, or infection with mycobacterium, can inhibit the prevalence of allergic disease [14, 28]. Shirakawa et al. demonstrated the existence of an inverse relationship between the total amount of serum IgE measured and the degree of delayed-type hypersensitivity (DHT) experienced, due to infection with a purified protein derivative of *Mycobacterium tuberculosis*

(PPD) in Japan [14]. In Japan, natural exposure to mycobacterium is common, which suggests that infection with *Mycobacterium* may inhibit the onset of atopy [14]. On the other hand, receiving a BCG vaccination before 6 months of age had no observed effect on the prevalence of atopy in Sweden, where the risk of tuberculosis is low [28]. Our finding that the footpad application of PPD had no effect may explain why percutaneous, but not local, BCG vaccination is ineffective in preventing airway allergy in humans living in non-endemic areas for tuberculosis. Erb et al. also demonstrated that intranasal infection with BCG is a more potent stimulus for eosinophil recruitment into airways, compared with intraperitoneal or subcutaneous infection with the pathogen [16]. Thus, direct application of PPD into the nose appears to be useful in preventing allergic rhinitis.

On the other hand, intranasal application of PPD did not regulate either the production of SEA-specific Abs or nasal eosinophilia in mice intranasally pre-sensitized with SEA. In addition, we confirmed that intranasal PPD treatment during the effector phase had no effect on allergic inflammation in mice systemically pre-sensitized with OVA (data not shown). Several reports showed that reversal of an established Th2 response is difficult and has not been effectively achieved by using recombinant cytokines [29,30]. Results presented here are consistent with the reports and suggest that the use of PPD might have limitations with regard to suppressing ongoing Th2 allergic inflammation. In contrast, Scott et al. demonstrated that the application of heat-killed BA down-regulated the secondary OVA-specific IgE response and enhanced the IgG2a response in OVA-pre-sensitized mice [15]. Yeung et al. also reported that heat-killed *Listeria monocytogenes* can act as an adjuvant and convert established Th2-dominated responses against keyhole limpet haemocyanin (KLH) into Th1-dominated responses [31]. In addition, Wang et al. showed that a single injection of heat-killed *Mycobacterium vaccae* into OVA-pre-immunized BALB/c mice suppressed an increase of serum IgE [32]. Therefore the suppressive effect of Th1-inducible intracellular pathogen itself and soluble protein

stimuli on the pre-existing and/or ongoing Th2 responses appears to be different.

There are several explanations of how intracellular pathogen but not soluble protein stimuli could promote the suppression of the pre-existing and/or ongoing Th2 responses. The most likely explanation is that the pathogen can exert the powerful and long-term suppression against allergic Th2 responses. For example, signals for INF- $\gamma$  could be detected for up to 48 h in the nose following a single intranasal application of PPD (Fig. 2a), whereas a single intranasal infection with BCG led to a significant increase of IFN- $\gamma$  production for up to 4 weeks [16].

A second possible explanation is that the intracellular pathogen can activate CD8<sup>+</sup> T cells, while exogenous soluble antigen usually can not lead to the activation. In fact, immunization with heat-killed *M. vaccae* results in the generation of CD8<sup>+</sup> T cells, which secrete IFN- $\gamma$  and enhance the production of IL-12 [33].

Another possible explanation is that the deposition, retention and clearance in murine tissues might differ between the particulated pathogen and soluble protein. For example, it is known that aerosolized pollutant was cleared from the respiratory tract by direct absorption into blood, while particulated pollutant was cleared by both blood absorption and mucociliary clearance followed by ingestion and faecal excretion [34]. In addition, protein stimuli appear to be more susceptible to degradation by mucous fluids containing enzymes such as lysozyme and peroxidase as compared with the pathogen itself [35].

In summary, our data demonstrate that intranasal application of PPD before and during sensitization suppresses the initiation of allergic rhinitis by skewing the nasal micro-environment towards Th1, although it has no exacerbative effect on ongoing allergic inflammation in pre-sensitized mice. As the use of local Th1-inducible stimuli has minimal side-effects, including a lack of granuloma formation, which has been observed to occur when the pathogen itself is used, clinical trials using local Th1-inducible stimuli should yield promising insight into the prevention of allergic rhinitis. In addition, further investigations should focus on the local application of other pathogen-derived stimuli, such as CpG oligodeoxynucleotide, as parental application of Th1-inducing stimuli may induce autoimmune diseases, most of which are controlled by Th1 immune responses [36, 37].

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