

Effect of Rolipram, a Phosphodiesterase IV Inhibitor, on Allergic Footpad Swelling using Various Adjuvants in Mice

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Abstract

We studied the effect of rolipram, a phosphodiesterase (PDE) IV inhibitor, on allergic footpad swelling in mice. For this study, varying adjuvants including complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) and Imject Alum (Alum) were used because the extent of antigen-specifically induced T helper type 1 (Th1) and Th2 responses had been shown to depend on adjuvants used. To induce allergic footpad swelling, we immunized mice with ovalbumin (OVA) emulsified in either CFA or IFA, dissolved in Alum or in phosphate-buffered saline (PBS) as a control (day 0), followed by subcutaneous injection of the antigen into footpads on day 21. Rolipram was given orally to the animals daily from days 0–20. Results showed that treatment with rolipram was followed by an increase in early swelling at 0.5 h and a decrease in late swelling at 6 and 24 h in the CFA group. In the IFA group, rolipram significantly enhanced swelling at, but not after, 30 min. In the Alum and the PBS groups, the PDE inhibitor failed to affect the OVA-specific footpad reaction at all times examined. Treatment of the CFA and IFA groups with rolipram significantly inhibited the production of the Th1 antibody anti-OVA immunoglobulin G2a (IgG2a), and the drug enhanced Th2 cell-dependent anti-OVA IgE production. In both groups, rolipram also enhanced the secretion of Th2 cytokines including interleukin-4 (IL-4) and IL-10. These findings suggest that rolipram may facilitate early allergic footpad swelling mediated by Th2 immune responses, while the late phase of swelling associated with Th1 responses may be attenuated by the PDE IV inhibitor.

Introduction

T helper type 1 (Th1) and Th2 cells produce distinct patterns of cytokines and play a role in the immune system [1, 2]. Th1 cells produce cytokines such as interleukin-2 (IL-2) and interferon- γ (IFN- γ) which are involved in cellular immunity, whereas Th2 cells produce cytokines such as IL-4, IL-5 and IL-6 which play a role in humoral responses. Th1 and Th2 cytokines modulate their functions each other, namely IFN- γ suppresses Th2 immune responses [3], whereas IL-4 [4] and IL-10 [5] down-regulate Th1 responses. Th1 and Th2 cytokines also play an important role in many diseases in humans. For instance, in rheumatoid arthritis (RA), IFN- γ is produced in joints of the patients [6], and neutralization of IFN- γ attenuates the severity of the disease [7]. On the other hand, in asthma, an increased expression of IL-4 mRNA is observed in sputum of patients [8], and neutralization of

IL-4 blocks the development of acute airway hypersensitivity in a murine asthma model [9].

Phosphodiesterase (PDE) IV is expressed in immune and inflammatory cells and is a key enzyme that degrades cAMP playing a role in inflammatory and immune reactions. The prevention of cAMP degradation by PDE IV inhibitors elevates the level of intracellular cAMP followed by suppression of inflammation and immune responses [10]. It has been reported that increased intracellular cAMP preferably inhibited production of Th1 cytokines more effectively than Th2 [11–14]. Moreover, treatment with PDE IV inhibitors of mice with experimental autoimmune neuritis induces attenuation of the disease as well as downregulation of Th1 cytokine production and up-regulation of Th2 cytokine production [15]. Thus, the drugs are regarded to be useful to treat Th1-dominant diseases. In contrast to the report, several studies indicated that

PDE IV inhibitors had a suppressive effect on Th2 responses and attenuated allergen-induced airway hyperresponsiveness [16, 17]. Recently, we reported that rolipram preferentially inhibited Th1 responses more effectively than Th2 responses and that the treatment with rolipram was able to inhibit Th1-mediated antigen [ovalbumin (OVA)]-induced arthritis in mice [18]. Therefore, we investigated the effect of the PDE IV inhibitor on allergic footpad swelling using varying adjuvants including CFA, IFA and Alum that had been shown to induce Th1 and Th2 immune responses to different extents [19].

Materials and methods

Materials. Male DBA/1J mice, 7–9 weeks of age, were used in all experiments. The animals were maintained in a temperature-controlled environment, with free access to standard rodent chow and water. All procedures in animal experiments were approved by the Animal Ethics Committee of Kobe Pharmaceutical University.

Immunization with OVA. One hundred micrograms of OVA (Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA) was dissolved in 50 µl of phosphate-buffered saline (PBS) and emulsified with an equal volume of complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, MI, USA) or incomplete Freund's adjuvant (IFA; Difco Laboratories). The same amount of OVA solution was also mixed with an equal volume of Imject Alum (Alum; Pierce Biotechnology, Rockford, IL, USA) or PBS. One hundred microlitres of the emulsions or solutions was injected subcutaneously at the base of the tail (day 0).

Administration of rolipram. Since our previous studies [18] showed that 5 mg/kg of rolipram modulated secretion of Th1 and Th2 cytokines, this dosage of the drug was used throughout the experiments. Rolipram (Sigma-Aldrich Fine Chemicals) was dissolved in 500 µl of saline containing 10% chremophor EL (Sigma-Aldrich Fine Chemicals) and orally administered daily (5 mg/kg) from days 0–20. Five hundred microlitres of saline containing 10% chremophor EL alone was given as a control. There was no significant difference in gaining body weights between control and rolipram-treated mice (data not shown).

Induction of allergic footpad reactions. On day 21, the immunized mice were challenged by subcutaneous injection of 10 µl of PBS containing 5 µg of OVA into the footpad of left hindpaw. The footpad of the right hindpaw was injected with 10 µl of PBS alone as a control. To evaluate the severity of the allergic reactions, the thickness of both footpads of the hindpaws was measured using a dial gauge calliper calibrated with 0.01 mm graduations. The net increase in footpad thickness attributable to the antigenic challenge was calculated by subtracting thickness of the footpad of the right hindpaw from that of the left hindpaw.

Measurement of antibodies to OVA. Sera were obtained on day 22 and heat inactivated at 56 °C for 30 min. Anti-OVA immunoglobulin G (IgG), IgG1 and IgG2a in sera were measured using enzyme-linked immunosorbent assay (ELISA) as described previously [20]. In brief, 96-well flat-bottom microlitre plates were incubated with 100 µl/well of OVA (100 µg/ml) at 37 °C for 1 h and washed three times with PBS containing 0.05% Tween-20. The wells were then blocked by incubation with 100 µl of PBS containing 1% casein (Sigma) at 37 °C for 1 h. After washing, the plates were incubated with 100 µl of a 1 : 5000 (for IgG and IgG1 measurement) or 1 : 500 (for IgG2a measurement) or 1 : 10 (for IgE measurement) dilution of each serum sample at 37 °C for 1 h. The plates were washed, and 100 µl/well of a 1 : 2000 dilution of alkaline phosphatase-labelled rat anti-mouse IgG (Sigma) or 1 : 1000 dilution of alkaline phosphatase-labelled anti-mouse IgG1 and IgG2a (PharMingen, San Diego, CA, USA) or 1 : 2000 dilution of anti-mouse IgE-biotin (LO-ME-2 BIOTIN; Funakoshi, Tokyo, Japan) was added and incubated at 37 °C for 1 h. For IgG measurement, after washing, 100 µl of 3 mM *p*-nitrophenylphosphate (Bio-Rad, Richmond, CA, USA) was added per well, and the plates were incubated in the dark at room temperature for 15 min and more. For IgE measurement, after washing, 100 µl of 1 : 500 dilution of alkaline phosphatase anti-biotin (Vector Laboratories, Burlingame, CA, USA) was added and incubated at 37 °C for 1 h. After washing, 100 µl of 3 mM *p*-nitrophenylphosphate (Bio-Rad) was added per well, and the plates were incubated in the dark at room temperature for 15 min. The absorbance was then measured at 405 nm by IMMUNO-MINI NJ-2300 (Nalge Nunc International K.K., Tokyo, Japan). The results are expressed as absorbance units at OD₄₀₅ ± SEM.

Measurement of cytokines. Spleens were removed on day 22, and cell suspension was prepared [21]. Erythrocytes in the cells were lysed with Tris-NH₄Cl. A total of 5×10^6 cells in 1 ml of RPMI-1640 (Sigma) containing 1 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol and 2% heat-inactivated autologous mouse serum were cultured in 24-well tissue culture plates either in medium alone or with 50 µg/ml OVA. Forty-eight hours later, supernatants were harvested and stored at –20 °C until assayed. Cytokine production was quantified by commercially available ELISA kit for IFN-γ, IL-10 (Endogen, Woburn, MA, USA) and IL-4 (BioSource International, Camarillo, CA, USA), and the net increase of cytokine production attributable to the *in vitro* antigen stimulation was calculated by subtracting the amount of cytokine in supernatant cultured without OVA from that with OVA.

Proliferation assay. A total of 5×10^5 of spleen cells in 0.1 ml of RPMI-1640 (Sigma) containing 1 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol and 2% heat-inactivated

autologous mouse serum were cultured with 50 µg/ml of OVA in each well of 96-well tissue culture plates. Seventy-two hours later, each well was pulsed with 0.5 µCi of tritiated thymidine, and the cells were cultured for another 17 h. The cultures were harvested onto fibreglass filters using multi-harvester and counted using standard liquid scintillation techniques. Results, expressed in cpm, are the average of triplicate cultures of cells pooled from five to six mice.

Statistics. To analyse data statistically, the Mann–Whitney *U*-test was used because sample sizes in our experiments were small, and therefore the normality obtained was poor.

Results

The effect of various adjuvants on OVA-specific allergic footpad swelling

As shown in Fig. 1A, injection of OVA to mice immunized with OVA and CFA resulted in marked footpad swelling, reaching peaks at 0.5 and 4 h. Moderate swelling was still observed at 24 h. Mice immunized with OVA plus IFA had a marked footpad swelling at 0.5 h and moderate swelling at 4 h. The swelling subsided almost completely by 24 h. In animals immunized with OVA, together with Alum or PBS, marked swelling was observed only at 0.5 h.

To examine the role of adjuvants in humoral and cellular immune responses, OVA-specific IgG and proliferative responses of spleen cells were measured, respectively. As shown in Fig. 1B, all adjuvants used enhanced anti-OVA IgG production, and the order of the efficacy of these adjuvants on anti-OVA IgG production was CFA > IFA > Alum > PBS. The same order of the efficacy of these adjuvants on OVA-specific lymphoid cell proliferation was also observed as shown in Fig. 1C.

The effect of rolipram on OVA-induced footpad swelling

The administration of rolipram to mice immunized with OVA and CFA resulted in significant increases in footpad swelling at 0.5–2 h (Fig. 2A). In contrast, the footpad reaction at 6 and 24 h was decreased by the drug. When IFA was used as an adjuvant, rolipram had a significantly enhanced effect on swelling only at 0.5 h (Fig. 2B). In the Alum and the PBS groups, the drug failed to affect the OVA-specific reaction (Fig. 2C,D).

The effect of rolipram on OVA-specific Th1 and Th2 immune responses associated with footpad swelling

Next, we examined whether the effect of rolipram on the OVA-specific footpad swelling was associated with production of antibodies to OVA and furthermore with the antigen-specific Th1 and Th2 immune responses. Anti-

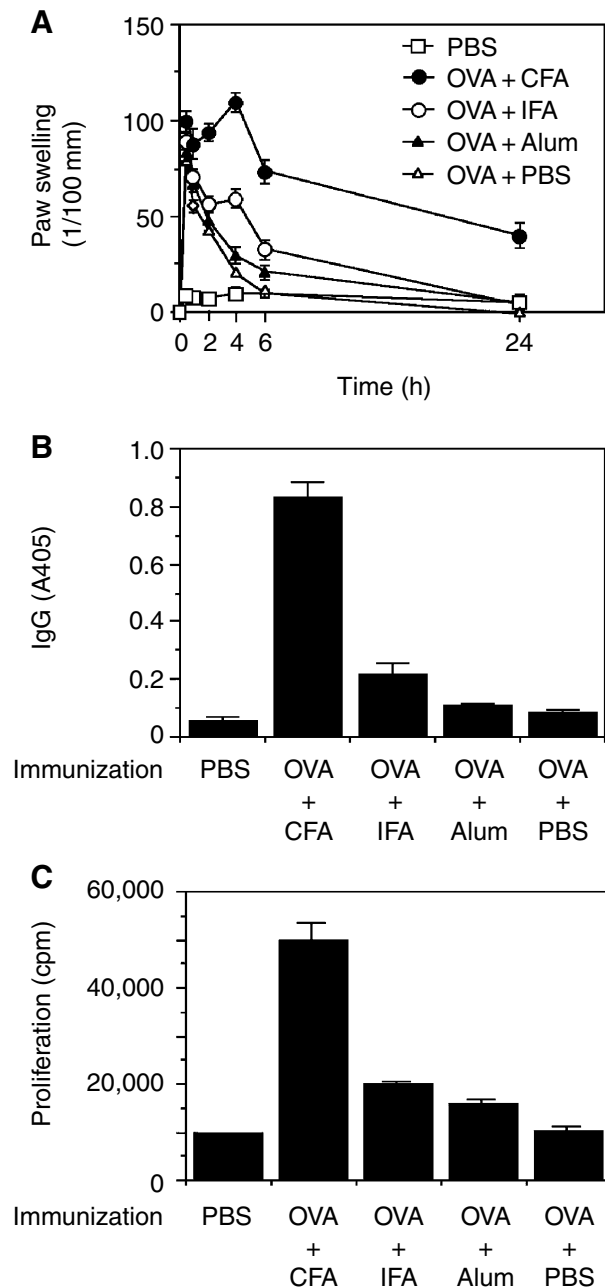


Figure 1 The effects of various adjuvants on ovalbumin (OVA)-specific footpad swelling, OVA-specific IgG production and OVA-specific cellular proliferative responses. Mice were immunized with OVA with the various adjuvants on day 0. Footpad swelling was induced by injection of OVA into the footpads on day 21, and anti-OVA IgG antibodies in sera and proliferative responses of spleen cells to OVA were measured on day 22 as described in *Materials and methods*. (A) Footpad swelling. Bars show mean $\pm$ SEM of five to seven mice. (B) Serum levels of anti-OVA IgG antibodies. (C) OVA-specific proliferative responses. Background counts of OVA-immunized lymphoid cells without OVA added were between 5000 and 6000 cpm.

OVA IgG2a and IFN- γ were measured as indicators of Th1 responses, while anti-OVA IgG1, IgE, IL-4 and IL-10 were measured as those of Th2 responses. As shown in Fig. 3A, treatment with rolipram appeared to suppress

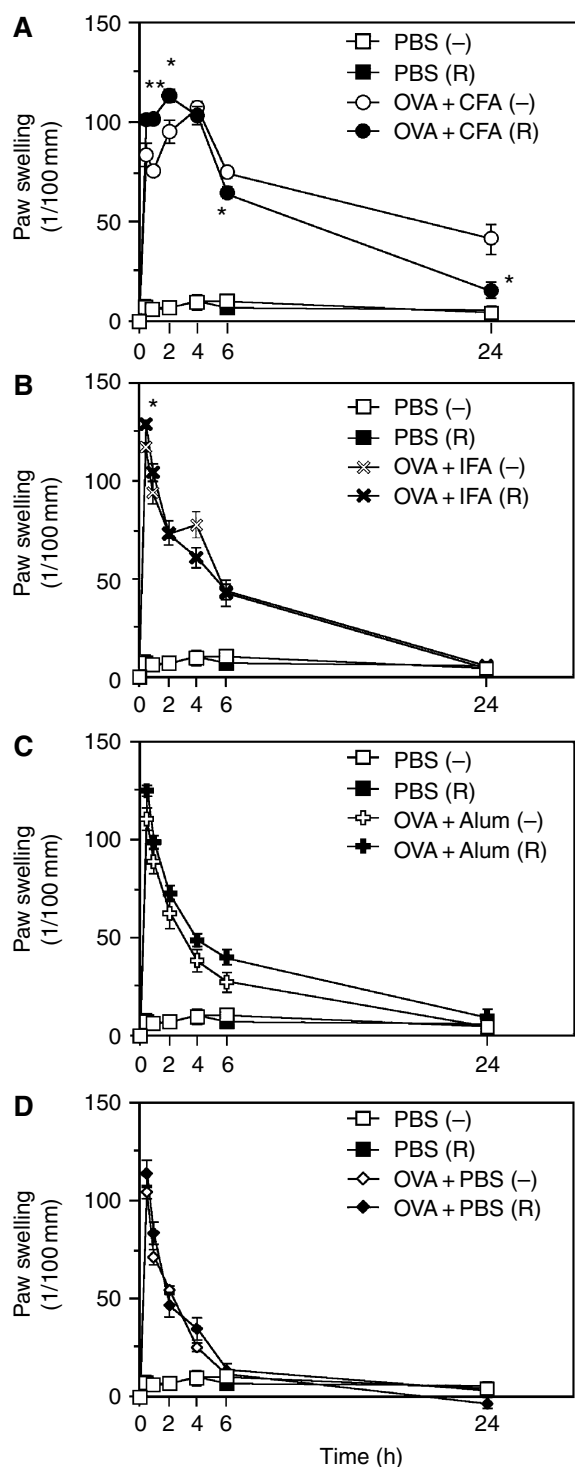


Figure 2 The effect of rolipram on allergic footpad swelling induced by ovalbumin (OVA) plus various adjuvants. Mice were immunized with OVA in CFA (A), IFA (B), Alum (C) and phosphate-buffered saline (PBS) (D) respectively on day 0. Five hundred microlitres of PBS with (R) or without (-) 5 mg/kg of rolipram was orally administered daily from days 0 to 20. PBS alone or rolipram was also orally given to non-immunized mice. On day 21, footpad swelling was induced by injection of OVA into footpads as described in *Materials and Methods*. The footpad swelling was measured at the times after the challenge injection. Bars show mean $\pm$ SEM of five to seven mice. * P < 0.05 versus corresponding group without rolipram.

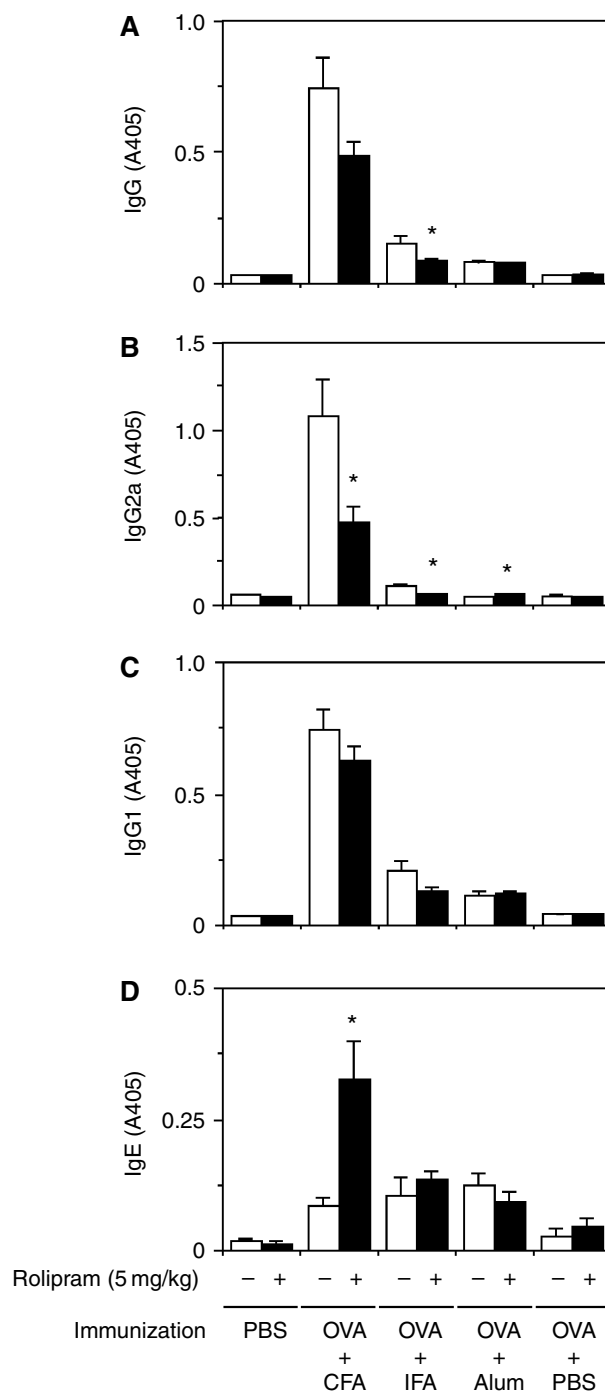


Figure 3 The effect of rolipram on anti-ovalbumin (OVA) IgG, IgG2a, IgG1 and IgE antibody production. Mice were immunized with OVA in complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), Alum and phosphate-buffered saline (PBS) on day 0. Five hundred microlitres of PBS with (■) or without (□) 5 mg/kg of rolipram was orally administered daily from days 0 to 20. On day 22, anti-OVA IgG, IgG2a, IgG1 and IgE antibodies in sera were measured as described in *Materials and Methods*. Bars show mean $\pm$ SEM of five to seven mice. * P < 0.05 versus corresponding group without rolipram.

anti-OVA IgG production when CFA and IFA were used as adjuvants. There was no effect of the PDE IV inhibitor on the antibody production in mice immunized with OVA together with Alum or PBS.

Both the CFA and the IFA groups treated with rolipram also showed decreased production of anti-OVA IgG2a (Fig. 3B). In contrast, in the Alum group, a small but significant enhancement of the isotype of antibody production by rolipram was seen. There was no significant effect of rolipram on the levels of anti-OVA IgG1 in all adjuvant-treated groups (Fig. 3C). On the other hand, treatment with rolipram stimulated production of anti-OVA IgE when CFA was used as an adjuvant, while the Alum group appeared to have less of the antigen-specific antibody (Fig. 3D).

The secretion of IFN- γ in the CFA and the IFA groups tended to be suppressed by treatment with rolipram (Fig. 4A). However, secretion of IL-4 appeared to be increased by rolipram when CFA was used as an adjuvant (Fig. 4B). Furthermore, secretion of IL-10 was also facilitated by the PDE IV inhibitor when CFA as well as other adjuvants was used (Fig. 4C).

Discussion

In the present study, we tried to clarify the effect of rolipram, a PDE IV inhibitor, on OVA-specific footpad swelling using various adjuvants including CFA, IFA and Alum that induce Th1 and/or Th2 immune responses to different extents. Th2 responses were shown by the production of anti-OVA IgG1 and IgE involved in acute inflammatory responses via mast cell activation [22], whereas Th1 responses were indicated as the production of anti-OVA IgG2a that could play a role in Arthus reaction via complement activation [23].

Antigenic challenge with OVA in the CFA group was followed by footpad swelling, with two strong peaks at 0.5 and 4 h, and moderate swelling was still observed at 24 h. In addition, the allergic responses were associated with marked production of all three isotypes of anti-OVA antibodies determined, i.e. IgE, IgG1 and IgG2a. Injection of OVA in the IFA group induced a strong early swelling at 0.5 h but a weak late swelling followed by complete remission by 24 h. This time course of footpad swelling was accompanied with a moderate production of anti-OVA IgE and IgG1 and a weak IgG2a production. Injection of OVA in the Alum group induced marked swelling, reaching a peak at 0.5 h associated with increased levels of anti-OVA IgE and IgG1. The marked swelling rapidly subsided by 6 h, and a much lower level of anti-OVA IgG2a was observed in this group. However, it is of note that there were comparable early peaks of footpad swelling at 0.5 h after challenge injection of OVA in all the CFA, IFA and Alum groups, with their similar production of anti-OVA IgE, indicating that anti-OVA IgE has a

major role in early footpad swelling via inducing a histamine-mediated anaphylactic reaction [24]. Thus, CFA, IFA and Alum appeared to induce Th2-mediated footpad responses, but Th1-dependent swelling in the footpad

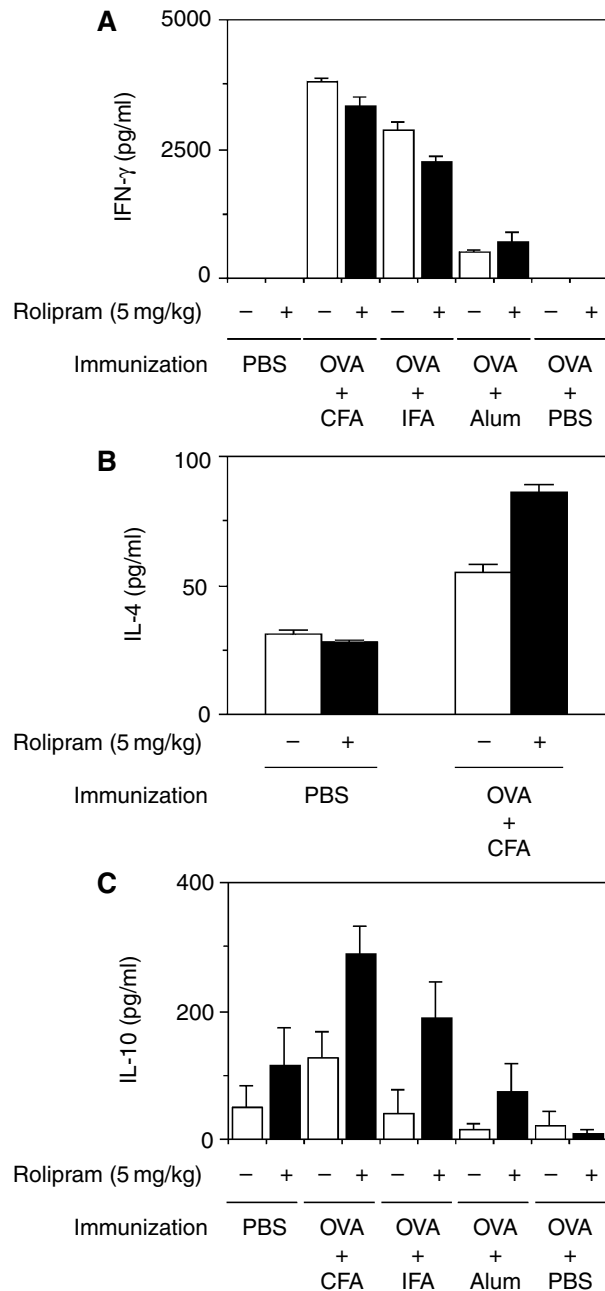


Figure 4 The effect of rolipram on interferon- γ (IFN- γ), IL-4 and IL-10 production by spleen cells. Mice were immunized with ovalbumin (OVA) in complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), Alum and phosphate-buffered saline (PBS) on day 0. Five hundred microlitres of PBS with (■) or without (□) 5 mg/kg of rolipram was orally administered daily from days 0 to 20. On day 22, IFN- γ (A), IL-4 (B) and IL-10 (C) were measured as described in *Materials and methods*. Bars show mean $\pm$ SEM of triplicated cultures of spleen cells pooled from five to seven mice.

appeared to be caused by CFA but not Alum. IFA might be in part involved in footpad swelling mediated by Th1 responses.

Treatment of the CFA group with rolipram over a period of 21 days was followed by significant increases in early footpad swelling at 0.5 h as well as increases in anti-OVA IgE production. In contrast, the drug treatment resulted in decreases in late footpad responses at 6 and 24 h associated with lower levels of anti-OVA IgG2a. There were similar trends in the IFA group treated with rolipram. These observations further supported that the early and late phases of footpad swelling appeared to be induced by anti-OVA IgE and IgG2a, respectively. It should be noted that rolipram treatment appeared to enhance the early footpad swelling via increased production of anti-OVA IgE but not via modulation of mast cell functions, because rolipram failed to affect a passive cutaneous reaction in guinea pigs [25] and mast cell degranulation *in vitro* [26, 27]. These results indicate that rolipram appeared to stimulate induction of Th2 immune responses but to inhibit Th1 responses.

In order to address the Th2 biasing effect of rolipram, we examined T-cell functions by measuring antigen-dependent secretion of IFN- γ as a Th1 cytokine and IL-4 and IL-10 as Th2 cytokines from spleen cells of mice treated with rolipram. In the CFA and the IFA groups, treatment with rolipram enhanced the secretion of IL-4 and IL-10, while IFN- γ secretion tended to be suppressed. As with the role of rolipram and other PDE IV inhibitors in Th1 and Th2 immune responses, it has been reported that they preferably inhibited Th1 responses more strongly than Th2 [28–30] via increases in intracellular cAMP [11, 31]. Thus, treatment with rolipram may shift Th1 to Th2 responses [28–30] by selective inhibition of Th1 functions followed by enhancement of Th2 functions. The drug appeared to downregulate IgG2a but upregulate IgE production.

On the other hand, the drug failed to modulate immune responses in the Alum group in which Th2 responses were well established but Th1 responses were not. This observation appears to be important because rolipram might exert its ability to shift immune responses from Th1 towards Th2 only when both Th1 and Th2 responses are well established. The PDE IV inhibitor might not be able to enhance Th2 responses when Th1 responses are not established.

Abbas *et al.* [15] demonstrated that rolipram had protective effects on experimental autoimmune neuritis, a model in which Th1 responses were predominantly involved. The protective effect of the PDE IV inhibitor on the neuritis was associated with downregulation of IFN- γ but upregulation of IL-4. Our results support those by Abbas *et al.*, although in the model we used, both Th1 and Th2 responses appeared to be involved to a similar extent. In contrast, Kanehiro *et al.* [16] reported

that PDE IV inhibitors suppressed allergen-induced airway hyperresponsiveness in which Th2 responses play a critical role. They also showed that the suppression of the airway disease by the drugs was associated with suppression of production of IL-4. In addition, there is a report demonstrating that PDE IV inhibitors prevented IL-4-induced IgE production in human peripheral blood mononuclear cells [32]. Thus, the results shown by Kanehiro *et al.* differed from ours because our results indicated that rolipram had no effect on Th2-dominant footpad reactions as shown in the Alum group.

In summary, PDE IV inhibitors including rolipram may upregulate Th2 responses, while Th1 responses may be downregulated by the drug when both Th1 and Th2 responses or Th1 but not Th2 responses are well established. Rolipram may lack such action or in certain conditions may suppress Th2 responses when Th2 but not Th1 responses are established, although further detailed studies are required before reaching conclusions.

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