Intracellular 3′,5′-Adenosine Cyclic Monophosphate Level Regulates House Dust Mite-Induced Interleukin-13 Production by T Cells from Mite-Sensitive Patients with Atopic Dermatitis

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We studied the relationship between cAMP and house dust mite-induced cytokine production in T cells from mite-sensitive patients with atopic dermatitis. T cells from atopic dermatitis patients secreted high level of interleukin-13 (mean 851.1 pg per ml) when cultured with autologous monocytes pulsed with *Dermatophagoides pteronyssinus* extract. *Dermatophagoides pteronyssinus*-induced interleukin-13 secretion was not detected in normal subjects. Adenylate cyclase inhibitor MDL 12,330A and cyclic nucleotide phosphodiesterase type 4 inhibitor rolipram blocked Dermatophagoides pteronyssinus-induced interleukin-13 secretion in atopic dermatitis T cells. In atopic dermatitis T cells, cAMP level rose at 5 min after *Dermatophagoides pteronyssinus* stimulus then decreased to the basal level at 1 h. MDL 12,330A blocked the *Dermatophagoides pteronyssinus*-induced cAMP elevation while rolipram blocked its reversal. In atopic dermatitis T cells, adenylate cyclase activity increased at 5 min after *Dermatophagoides pteronyssinus* stimulus, followed by the increase of cyclic nucleotide phosphodiesterase acvity at 15 min. In atopic dermatitis T cells, phospholipase C inhibitor ET-18-OCH3 blocked *Dermatophagoides pteronyssinus*-induced activation of adenylate cyclase, while rolipram, protein kinase A inhibitor H-89, and MDL 12,330A blocked the activation of cyclic nucleotide phosphodiesterase. These results suggest that *Dermatophagoides pteronyssinus* may first increase cAMP in atopic dermatitis T cells by activating adenylate cyclase via phospholipase C, and next decrease cAMP by activating cyclic nucleotide phosphodiesterase 4 via protein kinase A, which may be activated by adenylate cyclase-generated cAMP signal. These events are required for interleukin-13 response *Dermatophagoides pteronyssinus*. *Key words: adenylate cyclase/cyclic nucleotide phosphodiesterase*/Dermatophagoides pteronyssinus. *J Invest Dermatol 116:3–11, 2001*

House dust mite allergen plays an important part in the development of allergic diseases such as atopic dermatitis (AD) (Nakagawa *et al*, 1987; Wedderburn *et al*, 1993; Lu *et al*, 1998). The production of house dust mite-specific IgE antibodies is highly enhanced in atopic patients compared with nonatopic subjects, and this triggers allergic responses (Lu *et al*, 1998). It is reported that T cells from atopic patients respond to mite allergen and predominantly produce T helper-2 (Th2) cytokines such as interleukin (IL)–4, IL-5, or IL-6, which stimulate humoral immunity. In contrast, the mite-induced production of Th1 cytokines such as interferon (IFN) -γ, IL-2, or lymphotoxin, which stimulate cellular immune responses, is impaired in atopic T cells (Wierenga *et al*, 1991). It is also reported that *Dermatophagoides pteronyssinus* (Dp) -induced IL-13 production is enhanced in atopic donors (Lu *et al*, 1998; Kimura *et al*, 2000). IL-13 is a more recently defined cytokine and shares many functional properties with IL-4 (Minty *et al*, 1993). In particular, IL-13 induces B cell IgE production by isotype switching (Wedderburn *et al*, 1993; Lu *et al*, 1998). The precise mechanism for the allergen-induced IL-13 production, however, has not been defined. It is recently reported that 3′,5′-adenosine cyclic monophosphate (cAMP)-elevating agents inhibit ragweed allergen-induced IL-13 production in the allergen-specific T cell clones from atopic donors (Essayan *et al*, 1997). Cellular cAMP levels are controlled by the balance of adenylate cyclase (AC), which synthesizes cAMP, and cyclic nucleotide phosphodiesterase (PDE) which hydrolyzes cAMP (Robicsek *et al*, 1991; Iyengar, 1993). Thus cAMP elevation is induced by AC stimulators, such as prostaglandin E_2, forskolin, or cholera toxin, and by PDE inhibitors, such as theophylline or 3-isobutyl-1-methylxanthine (Anastassiou *et al*, 1992; Essayan *et al*, 1997). PDE is an isozymic

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Abbreviations: AC, adenylate cyclase; AD, atopic dermatitis; AP-1, activating protein-1; ATF-1, activation transcriptional factor-1; BAPTA/AM, 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl)ester; cAMP, 3′,5′-adenosine cyclic monophosphate; CREB, 3′,5′-adenosine cyclic monophosphate-responsive element-binding protein; Dp, *Dermatophagoides pteronyssinus*; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphorylcholine; H-89, *N*-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide; IP_3, inositol 1,4,5-triphosphate; MDL 12,330A, *cis*-*N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine hydrochloride; 8mmIBMX, 8-methoxymethyl-3-isobutyl-1-methylxanthine; PDE, cyclic nucleotide phosphodiesterase; PPD, purified protein derivative; Rp-8-Br-cGMPS, guanosine 3′,5′-cyclic monophosphorothioate, 8-bromo-, rp-isomer; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

family composed of various subtypes (Robicsek *et al*, 1991), and a previous study reported that a cAMP-specific PDE (PDE)4 inhibitor suppressed allergen-induced IL-13 production (Essayan *et al*, 1997).

It is known that the cAMP-elevating agents suppress phytohemagglutinin-induced or calcium ionophore/phorbol 12-myristate 13-acetate-induced production of Th1 cytokines such as IFN-γ or IL-2 (Anastassiou *et al*, 1992; Yoshimura *et al*, 1998). It is suggested that protein kinase A, activated by the binding of cAMP, may downregulate the transcriptional, or pretranscriptional or post-transcriptional site of Th1 gene expression (Anastassiou *et al*, 1992; Penix *et al*, 1996). In contrast, regarding the regulation of Th2 production by cAMP, conflicting results are reported. Lacour *et al* (1994) reported that cAMP-elevating agents upregulated IL-4 and IL-5 production of ionophore/phorbol 12-myristate 13-acetate-activated murine $CD4^+$ T cells, and suggested that cAMP may switch the T cell cytokine profile towards Th2. On the other hand, Borger *et al* (1996) reported that cAMP-elevating agents suppressed IL-4 production of concanavalin A-activated human T cells or anti-CD3/anti-CD28-activated human T cells. Essayan *et al* (1995) reported that cAMP-elevating agents did not alter ragweed antigen-induced IL-4 production by human peripheral blood mononuclear cells. Possibly the modulatory effects of cAMP on Th2 production may vary depending on the T cell sources used and the stimulation conditions applied. IL-13 is preferentially expressed in Th2-type cells; however, it is also expressed in some Th1 clones or Th0 clones that produce IL-2 or IFN-γ together with IL-4 or IL-5 (De Waal Malefyt *et al*, 1995). It is thus possible that cAMP may regulate IL-13 production differently from other prototypic Th2 cytokines, such as IL-4 or IL-5.

In this study, we first examined mite allergen-induced cytokine production in T cells from mite-sensitive AD patients and normal subjects. The allergen potently and specifically induced IL-13 production in the patients' T cells. We then analyzed the regulatory effect of cAMP on the mite-induced IL-13 production with regard to the activities of AC and PDE.

MATERIALS AND METHODS

Patients and controls We studied 20 patients with mild to severe AD [10 men and 10 women, age 25.2 ± 1.4 y (mean ± SEM)], diagnosed according to the criteria by Hanifin and Rajka (1980). Their disease severity was scored according to the grading by Rajka and Langeland (1989), and the score was 5.0 ± 0.4 (mean ± SEM). Serum total IgE and IgE antibody specific for Dp were determined by chemiluminescence enzyme immunoassay using the LUMIWARD automated allergy system (Shionogi, Osaka, Japan). All patients showed elevated serum Dp-specific IgE (≥ 0.7 IU per ml; chemiluminescence enzyme immunoassay class ≥ 2). The Dp-specific IgE value of the patients was 31.1 ± 7.1 IU per ml and total IgE value was 4708 ± 1607 U per ml (mean ± SEM). The patients' peripheral blood eosinophil count was 939 ± 153 per μl, and serum lactic dehydrogenase value was 341 ± 26 U per l (mean ± SEM). At the time of the study, 16 of 20 patients were treated with topical corticosteroids that were ranked mild to very strong. No patients were taking systemic corticosteroids, anti-allergic or anti-histaminic drugs, or receiving desensitization immunotherapy. Nonatopic healthy volunteers [seven men and eight women, age 26.4 ± 2.1 y (mean ± SEM)] were studied as controls. None of the controls had symptoms indicative of allergic diseases, or receiving medication, and their serum Dp-specific IgE value was < 0.35 IU per ml; chemiluminescence enzyme immunoassay class 0. At the time of the investigation, all the patients and controls did not have active infection by fungi, viruses, or bacteria, and consumed no beverage containing caffeine or methylxanthine for at least 8 h before drawing blood. All the patients and controls were informed of the objectives and methods of this study, and consented to participate.

Reagents The crude extract from Dp was purchased from Cosmo Bio (Tokyo, Japan). *Mycobacterium tuberculosis* purified protein derivative (PPD) was from Evans Medical Ltd (Horsham, Sussex, U.K.). Rolipram, 8-methoxymethyl-3-isobutyl-1-methylxanthine (8mmIBMX), milrinone, *cis*-*N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine hydrochloride (MDL 12,330A) were obtained from Calbiochem (La Jolla, CA), and were dissolved in dimethylsulfoxide as 10 mM stock solutions. 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphorylcholine (ET-18-OCH_3) was from Calbiochem and was dissolved in ethanol as 10 mM stock solution. Calphostin C, *N*-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), and 1,2-*bis*(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl)ester (BAPTA/AM) were obtained from Calbiochem, and were dissolved in dimethylsulfoxide as 10 mM stock solutions. Guanosine 3′,5′-cyclic monophosphorothioate, 8-bromo-, rp-isomer (Rp-8-Br-cGMPS) from Calbiochem was dissolved in distilled water as 10 mM stock solution. All the stock solutions above were kept in the dark until used.

Preparation of monocytes and T cells Peripheral blood mononuclear cells were isolated by centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden) as described (Boyum, 1968), and were allowed to adhere to plastic dishes for 2 h at 37°C. From the adherent cells, $CD3^-$, $CD19^-$, and $CD56^-$ cells were isolated by negative selection using immunomagnetic beads (Dynal, Great Neck, NY) as described (Gee *et al*, 1987), and used as monocytes. This monocyte population was > 97% $CD14^+$, and the contamination of $CD3^+$, $CD19^+$, or $CD56^+$ cells was < 1% by flow cytometry. From the nonadherent cells, $CD56^-$ cells were isolated by negative selection using immunomagnetic beads (Dynal), and were incubated with neuraminidase-treated sheep erythrocytes as described (Farrant *et al*, 1985). From the rosette-forming cells, $CD14^-$ and $CD19^-$ cells were isolated by immunomagnetic negative selection, and were used as T cells. This T cell population was > 98% $CD3^+$, and the contamination of $CD14^+$, $CD19^+$, or $CD56^+$ cells was < 2%.

Measurement of cytokines We used endotoxin-, hormone-, and serum-free medium, 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's Nutrient Mixture F-12 (Sigma, St. Louis, MO), supplemented with 365.1 mg per l L-glutamine (Gibco/BRL, Grand Island, NY). Monocytes were irradiated (25 Gy) and used as antigen-presenting cells. Monocytes (5×10^4) were seeded in triplicate on to 96-well plates and cultured with or without Dp extract or prototypic Th1-antigen PPD (each 10 μg per ml) in total 200 μl medium for 3 h at 37°C (antigen pulse). The plates were centrifuged, and washed three times with medium. Autologous T cells (2×10^5) were then added and cultured with Dp- or PPD-pulsed or nonpulsed monocytes in total 200 μl medium at 37°C in an atmosphere of 5% CO_2 in air for 48 h. The culture supernatants were harvested and stored at −70°C until used. The activity of IFN-γ, IL-4, or IL-13 in the culture supernatants was measured by an enzyme-linked immunosorbent assay kit (Biosource, Tokyo, Japan) according to the manufacturer's instruction. The sensitivity of the assay for IFN-γ, IL-4, or IL-13 was 4, 3, or 12 pg per ml, respectively. The antigen-induced cytokine secretion of T cells was calculated as cytokine concentration of the culture with antigen-pulsed monocytes minus that of the culture with nonpulsed monocytes (background secretion). We considered positive response to antigen if the antigen-induced cytokine secretion was > 10 pg per ml and more than background.

For the analysis of cytokine mRNA expression, reverse transcription–polymerase chain reaction (reverse transcription–PCR) was performed. Nonadherent cells (> 95% $CD3^+$, < 2% $CD14^+$) were harvested from the combined culture of T cells and monocytes after 12 h, and were used as T cells. Total RNA was extracted from the harvested T cells, and was reverse transcribed as described (Kanda, 1999). Primer sequences for cytokines and for the internal control β-actin as well as PCR conditions are described elsewhere (Jung *et al*, 1996; Jaffar *et al*, 1999; Kanda, 1999). The PCR products were analyzed by electrophoresis on 2.5% agarose gels and stained with ethidium bromide. The intensity of the band for each cytokine PCR product was determined by densitometry (Hoefer Scientific Instruments, San Francisco, CA) and the ratio relative to β-actin product was calculated.

Measurement of cAMP To measure cAMP level of T cells at various time points, T cells harvested as described above were lyzed with ethanol, and the lysates were centrifuged and the supernatants were dried under vacuum. The dried samples were dissolved in acetate buffer (pH 5.8), and cellular cAMP contents were measured by enzyme-linked immunosorbent assay (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. The sensitivity of the assay was 12 fmol per assay well. At each time point, the number of the harvested cells was recounted in parallel and cAMP level was presented as pmol per 10^6 cells.

Proliferation assays T cells were cultured in triplicate with autologous irradiated antigen-pulsed or nonpulsed monocytes as described above for 40 h, and 0.5 μCi per well of [^{3}H]thymidine (Amersham) was added and the cells were incubated for an additional 8 h prior to harvest. The incorporation of [^{3}H]thymidine was assayed by liquid scintillation.

Generation of short-term Dp-specific Th1 cell line Dp-specific Th1 cell lines were generated from peripheral blood mononuclear cells as described (Del Preto *et al*, 1991). Briefly, peripheral blood mononuclear cells from AD or normal subjects were cultured with Dp (10 μg per ml) in 24-well-bottomed plates for 12 d, the last 7 d in the presence of 20 U per ml human recombinant IL-2 (Cetus, Emeryville, CA). Viable T blasts were harvested and tested for the Dp-specific proliferation and cytokine secretion using irradiated autologous monocytes as antigen-presenting cells. Dp-specific Th1 cell lines were defined as the T blasts showing Dp-specific proliferation (Dp-induced increase of [^{3}H]thymidine uptake $\geq$ 10,000 cpm) and high IFN-γ response to Dp ($\geq$ 500 pg per ml) without IL-4 or IL-13 response to Dp (< 10 pg per ml). Dp-specific Th1 cell lines could be generated from three of 10 normal donors, whereas they could not be generated from the 10 AD patients.

Measurement of PDE activity At various time points of the culture, T cells harvested as described above were sonicated at 2×10^7 per ml, and the cell lysate was assayed for PDE activity as described (Robicsek *et al*, 1991) using 1 μM of [2,8–^{3}H]cAMP (30 Ci per mmol) (Amersham) as a substrate. Each assay was performed in triplicate, and results were presented as pmol cAMP hydrolyzed per min per mg protein.

Measurement of membrane AC activity The T cell lysate obtained as described above was centrifuged at 23,600 × *g*, and the pellet was used as the membrane fraction as described (Choi *et al*, 1992). The AC activity of the membrane fraction was assayed as described (Salomon *et al*, 1974) using 1 mM [α-^{32}P]adenosine triphosphate (30 Ci per mmol) (Amersham) as a substrate. Each assay was performed in triplicate, and results were presented as pmol cAMP formed per min per mg protein.

Measurement of inositol 1,4,5-triphosphate (IP_3) T cells were incubated in a medium containing 10 mM LiCl with autologous antigen-pulsed or nonpulsed monocytes for 3 min. Nonadherent T cells were harvested as described above, and after washing, soluble phosphates were extracted with 0.2 vol. of ice-cold 20% $HClO_4$ for 20 min. After centrifugation at 2000 × *g* at 4°C for 15 min the supernatants were neutralized with Tris–KOH. IP_3 was quantified with a radioreceptor assay system from Amersham, and was presented as pmol per 10^6 cells.

Statistical analyses Mann–Whitney *U* test was used for the comparison of mean amounts in AD and normal groups in **Fig 1**. Fisher's exact test was used for the difference in the incidence of positive cytokine response between the two groups in **Fig 1**. For the data in **Fig 3**, one-way analysis of variance with Dunnet's multiple comparison test was used. For the data in **Figs 2** and **8** and **Table I**, one-way analysis of variance with Scheffe's multiple comparison test was used. Spearman's correlation coefficient was determined in the data for **Table II** and **Fig 6**. A value of $p < 0.05$ was considered significant.

RESULTS

Cytokine production in response to Dp T cells from Dp-sensitive AD patients and normal donors were cultured with autologous Dp-pulsed or nonpulsed monocytes, and IL-13, IL-4, and IFN-γ secretion were measured. The Dp-induced IL-13 secretion was markedly higher in AD patients (mean ± SEM, 851.1 ± 249.3 pg per ml) than that in controls (–0.1 ± 1.3 pg per ml, $p < 0.0001$) (**Fig 1*a***). None of the normal subjects showed a positive IL-13 response to Dp, and the incidence of the positive response was higher in AD patients than that in controls ($p < 0.0001$). We analyzed the correlation of Dp-induced IL-13 secretion in the AD patients with their clinical parameters (**Table II**). Dp-induced IL-13 secretion strongly correlated with Dp-specific IgE values and did not correlate with other parameters, disease severity score, total IgE, eosinophil count, or lactic dehydrogenase value. This indicates that Dp-induced IL-13 secretion may contribute to the synthesis of Dp-specific IgE antibody *in vivo*. In contrast, Dp-induced IL-4 and IFN-γ secretion were very low both in AD and normal groups (maximally < 20 pg per ml). There were no significant differences in the incidence or the mean amount of IL-4 (**Fig 1*b***) and IFN-γ (**Fig 1*c***) secretion between the two groups. The further enhanced IL-4 or IFN-γ secretion induced by Dp was not detected either in AD or normal T cells at the culture period of 6, 12, 24, or 72 h (data not shown).

In contrast to Dp, PPD induced high IFN-γ secretion both in AD (mean ± SEM, 233.1 ± 38.2 pg per ml) and normal (225.0 ± 40.1 pg per ml) groups, and there was no difference in the incidence or mean amount (**Fig 1*f***). PPD-induced IL-13 (**Fig 1*d***) and IL-4 (**Fig 1*e***) secretion was very low (maximally < 30 pg per ml) in both AD and normal groups, and there were no differences in the incidence or mean amount.

We also examined the antigen-induced cytokine mRNA expression. When nonadherent cells were harvested from the combined culture of T cells and monocytes, this population was > 95% $CD3^+$ and < 2% $CD14^+$. The cytokine mRNA expression in this nonadherent population was analyzed by reverse transcription–PCR (**Fig 2**). The results in mRNA expression were parallel to those in protein secretion; Dp induced IL-13 mRNA expression only in AD T cells, whereas PPD induced IFN-γ mRNA expression equivalently in AD and normal T cells. Both Dp and PPD only slightly increased IL-4 mRNA levels in AD and normal T cells and the difference from controls without antigens was not significant (**Fig 2*b***), indicating that the low levels of IL-4 secretion (**Fig 1*b*, *e***) may not be due to consumption by T cells.

Thus Dp specifically and greatly induced IL-13 production in AD T cells, whereas it induced very low levels of IFN-γ and IL-4 production. The subsequent experiments will be thus focused on Dp-induced IL-13 production in AD T cells. We aimed to clarify the mechanism for the Dp-induced IL-13 production regarding the cAMP-related signaling pathways.

The modulation of Dp-induced IL-13 secretion by the enzymes related to cAMP metabolism We first examined the involvement of cAMP-synthesizing AC and cAMP-hydrolyzing PDE in Dp-induced IL-13 secretion of AD T cells. T cells were preincubated with PDE or AC inhibitors before the culture with Dp-pulsed monocytes, and their inhibitory effects on IL-13 secretion were compared. As shown in **Fig 3(*a*)** AC inhibitor MDL 12,330A concentration dependently inhibited Dp-induced IL-13 secretion ($IC_{50} = 0.2$ μM), and complete inhibition was detected at 5 μM. Rolipram, an inhibitor of cAMP-specific PDE

Table I. The IP_3 increase and proliferation in response to Dp or PPD[a]

Antigen	IP_3 (pmol per 10^6 cells)		Proliferation (cpm)	
	AD (n = 5)	Nonatopic (n = 3)	AD (n = 5)	Nonatopic (n = 3)
None	0.223 ± 0.071	0.297 ± 0.093	1003 ± 210	2505 ± 526
Dp	0.631 ± 0.143[b]	1.095 ± 0.283[b]	5975 ± 1,023[b]	22 523 ± 3,432[b]
PPD	0.682 ± 0.165[b]	0.325 ± 0.111	6585 ± 1,503[b]	2925 ± 663

[a]T cells from AD patients and Dp-specific Th1 cell lines from nonatopic donors were cultured with autologous Dp-pulsed or PPD-pulsed or nonpulsed monocytes, and IP_3 level was measured at 3 min, whereas proliferation was assayed at 48 h as described in *Materials and Methods*. The data are mean ± SEM.
[b]$p < 0.01$ *versus* control cultures with antigen-nonpulsed monocytes.

Table II. The correlation of Dp-induced IL-13 secretion with clinical parameters in AD patients (n = 20)

Parameters	r_s[a]	p
Dp-specific IgE	0.923	<0.0001
Total IgE	–0.120	0.6019
Eosinophil count	0.284	0.2153
Lactic dehydrogenase value	–0.201	0.3812
Disease severity score	–0.087	0.7037

[a]Spearman's correlation coefficient.

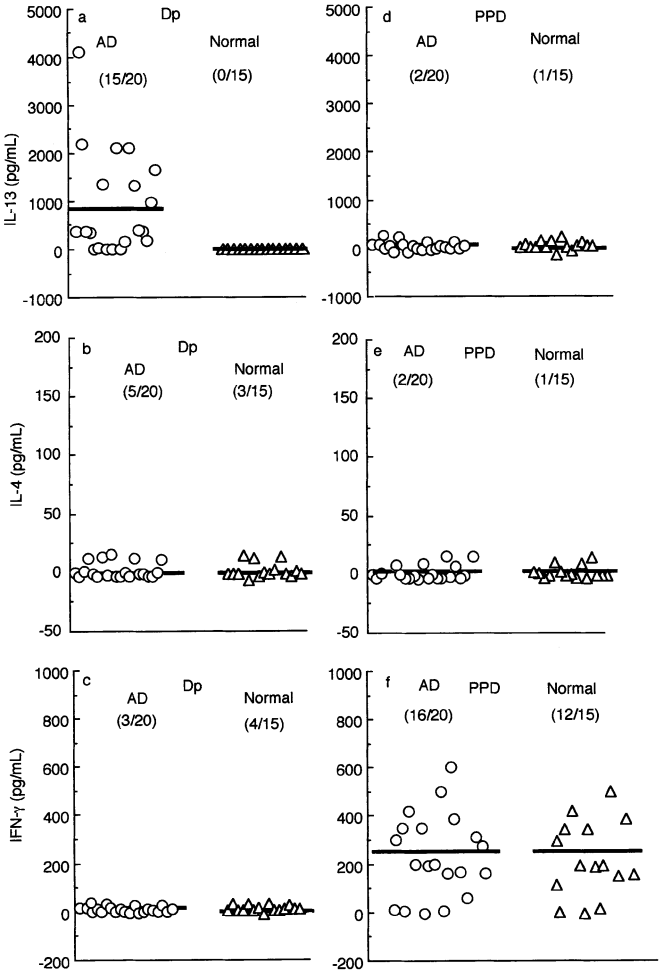

Figure 1. Dp- or PPD-induced IL-13, IL-4, and IFN-γ secretion of T cells from AD patients and normal subjects. (*a*, *d*) IL-13, (*b*, *e*) IL-4, (*c*, *f*) IFN-γ Each point represents the mean of triplicate cultures from individual donors. Bars show the group means. Values in parentheses are the number of donors with positive cytokine response/total donor number. The following are background cytokine secretion without antigens in AD patients *versus* normal subjects and p-value in the comparison by Mann–Whitney U test: IL-13 52.9 ± 8.9 (mean ± SEM) *versus* 22.1 ± 3.6 pg per ml, $p = 0.0037$; IL-4 4.2 ± 0.6 *versus* 4.1 ± 0.7 pg per ml, $p = 0.8752$; IFN-γ 8.1 ± 1.0 *versus* 7.1 ± 1.4 pg per ml, $p = 0.6935$.

(PDE4) also completely inhibited Dp-induced IL-13 secretion ($IC_{50} = 0.1\ \mu M$). In contrast, neither cGMP-inhibited PDE (PDE3) inhibitor milrinone, nor Ca^{2+}/calmodulin-dependent PDE (PDE1) inhibitor 8mmIBMX suppressed the Dp-induced IL-13 secretion. These results suggest that both AC and PDE4 may be required, whereas PDE1 and PDE3 may not be required for the Dp-induced IL-13 secretion in AD T cells.

In comparison, the effects of AC or PDE inhibitors were assessed on antigen-induced IFN-γ secretion. As shown in **Fig 3**(***b***), PPD-induced IFN-γ secretion in AD T cells identical to those in **Fig 3**(***a***) was inhibited by rolipram but not by MDL 12,330A, and the similar results were obtained in normal donors' T cells (data not shown). To know the AC or PDE inhibitors' effects on Dp-induced IFN-γ secretion, we tried to generate short-term Dp-specific Th1 cell lines as described in *Materials and Methods*, and the cell lines were obtained from normal donors. The attempt to generate Dp-specific Th1 cell lines from AD patients was unsuccessful (data not shown). As shown in **Fig 3**(***c***), Dp-induced IFN-γ secretion in Dp-specific Th1 cell lines from normal donors was completely blocked by rolipram, but not by MDL 12,330A. These results suggest that both Dp-induced IFN-γ secretion and PPD-induced IFN-γ secretion may involve PDE4 but not AC.

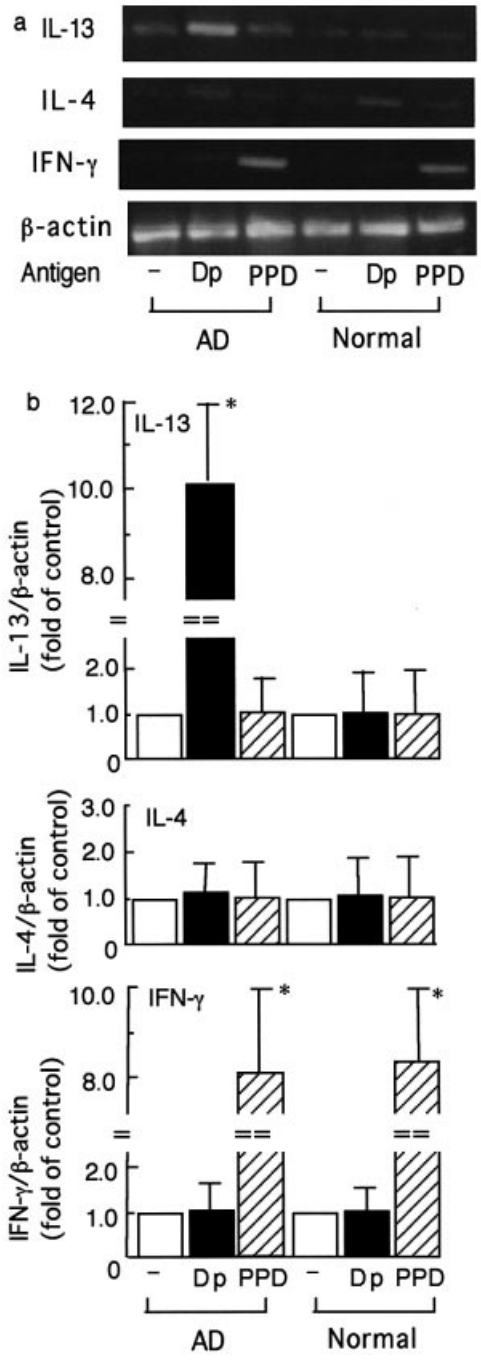

Figure 2. Dp- or PPD-induced cytokine mRNA expression. mRNA expression for IL-13, IL-4, and IFN-γ was analyzed by reverse transcription–PCR in T cells after 12 h culture with autologous antigen-pulsed or nonpulsed monocytes. (*a*) Representative results are shown from a single culture event of an AD patient's and normal donor's T cells. (*b*) Densitometry data are depicted for all AD patients (n = 20) and normal donors (n = 15) for each cytokine. The intensity ratio relative to β-actin PCR band was calculated for each cytokine band, and was normalized for control value without antigens, which is defined as 1.0. *$p < 0.05$ *versus* control without antigens.

Kinetics of intracellular cAMP level in AD T cells cultured with Dp-pulsed monocytes We next tried to examine the kinetics of cAMP level of T cells after incubation with antigen-pulsed monocytes. As shown in **Fig 4**, cAMP level of AD T cells increased 4-fold above the basal level at 5 min of the culture with Dp-pulsed monocytes, then decreased, and returned to the basal level at 1 h. The early rise of cAMP was blocked by AC inhibitor MDL 12,330A, suggesting that AC activity may be enhanced in the

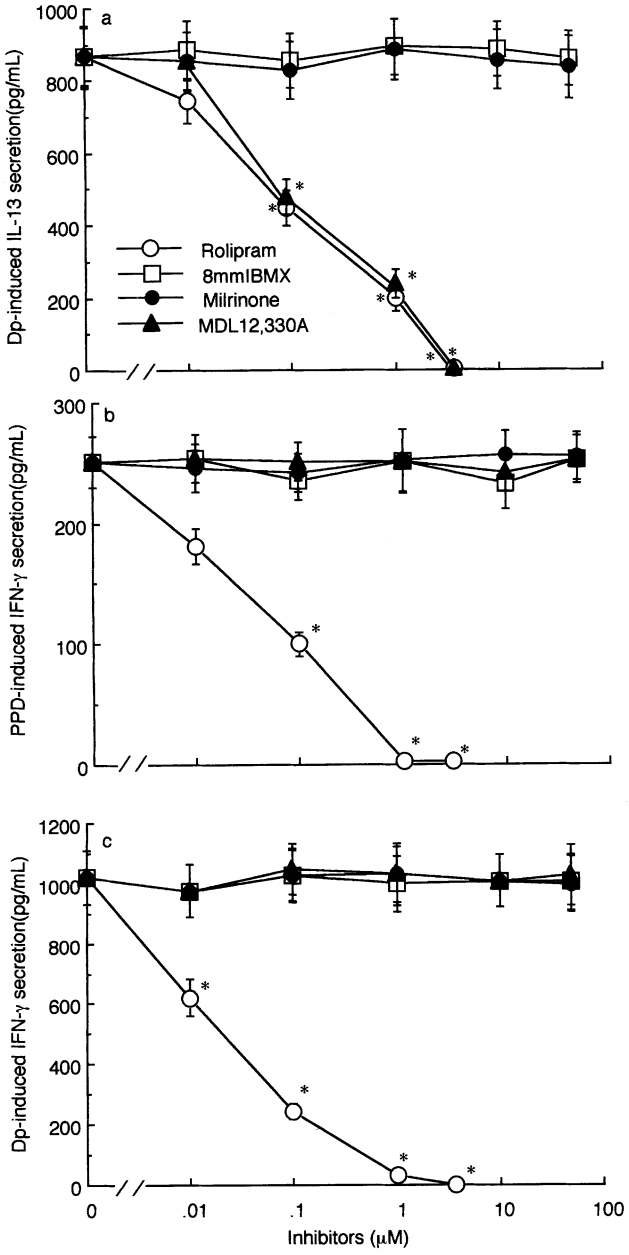

Figure 3. The inhibitory effects of AC and PDE inhibitors on antigen-induced cytokine secretion. (*a*) T cells from five AD patients (two men and three women) were preincubated for 30 min with medium alone or with medium containing AC or PDE inhibitors at indicated doses, washed and then added to the autologous Dp-pulsed or nonpulsed monocytes pre-adhered to 96-well plates. IL-13 secretion was examined after 48 h. Background IL-13 secretion was 51.3 ± 7.8 pg per ml (mean ± SEM, n = 5). (*b*) T cells identical to those in (*a*) were similarly treated except that PPD was used as an antigen and IFN-γ secretion was tested. Background IFN-γ secretion was 9.0 ± 1.3 pg per ml (mean ± SEM, n = 5). (*c*) Dp-specific Th1 cell lines from normal donors (n = 3, one man and two women) were similarly treated using Dp as an antigen, and IFN-γ secretion was tested. Background IFN-γ secretion was 30.0 ± 6.3 pg per ml (mean ± SEM, n = 3). Values are the mean ± SEM. * $p < 0.05$ *versus* control cultures without inhibitors.

early phase (≤ 5 min). In contrast, PDE4 inhibitor rolipram retained the elevated cAMP level, suggesting that PDE4 may reverse the early rise of cAMP. As opposed to Dp, Th1 antigen PPD did not alter cAMP level in the identical AD T cells. Besides Dp did not alter cAMP level in a short-term Dp-specific Th1 cell line from a normal donor. These results suggest that the alteration

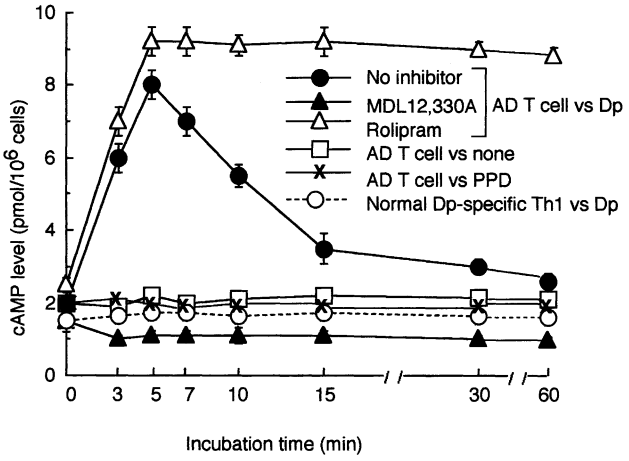

Figure 4. Kinetics of intracellular cAMP level of T cells after Dp or PPD stimulus. T cells from an AD patient were preincubated for 30 min with medium alone or with medium containing MDL 12,330A or rolipram (each 5 μM). The cells were washed and then added to the autologous Dp- or PPD-pulsed or nonpulsed monocytes. Dp-specific Th1 cell line from a normal donor was similarly treated in parallel. Intracellular cAMP level of T cells was analyzed at the indicated time points of the culture. The mean ± SD of triplicate cultures is shown. The data are representative of three separate experiments using three different AD patients' T cells and three different Th1 cell lines from normal donors.

of cAMP level after antigen stimulus may be specific to Dp-stimulated AD T cells and may not occur in PPD-stimulated AD T cells or Dp-stimulated Dp-specific Th1 cell line from a normal donor. We then studied the kinetics of AC and PDE activities in the AD T cells after culture with Dp-pulsed monocytes.

Kinetics of AC and PDE activities in AD T cells cultured with Dp-pulsed monocytes The AC activity in AD T cells was increased and peaked at 5 min of the culture with Dp-pulsed monocytes, then decreased, and returned to the basal level at 30 min (**Fig 5*a***). The transient AC activation was specifically blocked by AC inhibitor MDL 12,330A, and not by PDE4 inhibitor rolipram. Th1 antigen PPD did not induce the activation of AC in the identical AD T cells. Dp did not induce the activation of AC in the Dp-specific Th1 cell line from a normal donor. Thus the antigen-induced activation of AC may be specific to Dp-stimulated AD T cells.

On the other hand, PDE activity of AD T cells was also enhanced by the Dp stimulus (**Fig 5*b***), although the enhancement occurred later than that of AC (**Fig 5*a***). The PDE activity increased and was peaked at 15 min, then gradually decreased, and returned to the basal level at 1 h. Rolipram blocked the Dp-induced activation of PDE, suggesting that PDE4 may be activated among PDE subtypes. Preincubation with MDL 12,330A suppressed the Dp-induced activation of PDE. The suppression by MDL 12,330A may not be due to the direct inhibition of PDE enzyme as the direct addition of MDL 12,330A to the PDE assay did not reduce the PDE activity as compared with controls (data not shown). As MDL 12,330A blocked the activation of AC prior to that of PDE (**Fig 5*a***), it is indicated that the initial activation of AC may be required for the following activation of PDE. Th1 antigen PPD did not induce the activation of PDE in AD T cells. Dp did not induce the activation of PDE in the Dp-specific Th1 cell line from a normal donor. We then analyzed the correlation of the Dp-induced AC or PDE activation with Dp-induced IL-13 secretion. Dp-induced IL-13 secretion strongly correlated both with the Dp-induced increase in AC activity (**Fig 6*a***) and with that of PDE (**Fig 6*b***). These indicate that both the activation of AC and PDE by Dp may contribute to IL-13 induction by Dp.

The relationship of Dp-induced T cell receptor (TCR) stimulation with AC and PDE activation The antigenic

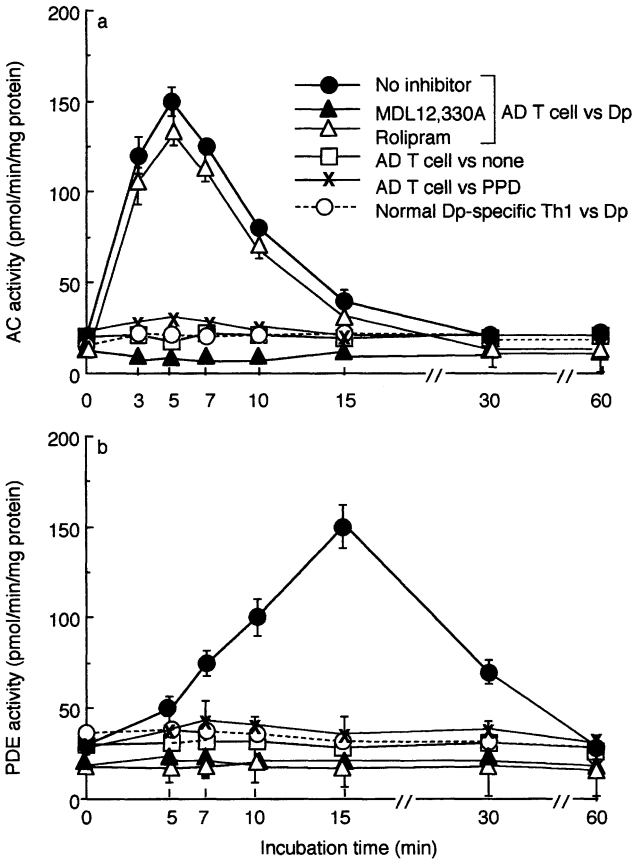

Figure 5. Kinetics of AC and PDE activity of T cells after Dp or PPD stimulus. (*a*) AC activity; (*b*) PDE activity. T cells from an AD patient and Dp-specific Th1 cell line from a normal donor, which are identical to those in **Fig 4**, were treated as described in **Fig 4** legend. The AC and PDE activities of the T cells were analyzed at the indicated time points of the culture. Data are the mean ± SD of triplicate assays. The data are representative of three separate experiments using three different AD patients' T cells and three different Th1 cell lines from normal donors.

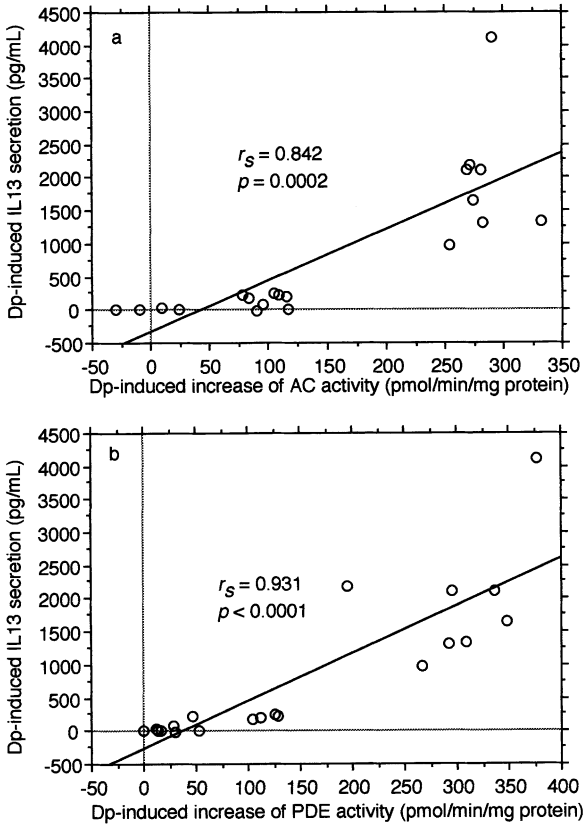

Figure 6. Relationship between Dp-induced IL-13 secretion and Dp-induced increase of AC activity or that of PDE activity in T cells from AD patients. (*a*) AC activity; (*b*) PDE activity. T cells were isolated from 20 AD patients identical to those in **Fig 1**. AC activity of the T cells was analyzed at 5 min, that of PDE was analyzed at 15 min, and IL-13 secretion was analyzed at 48 h of the culture with autologous Dp-pulsed or nonpulsed monocytes, respectively. The Dp-induced increase of AC or PDE activity was calculated as AC or PDE activity of T cells cultured with Dp-pulsed monocytes minus that of T cells with nonpulsed monocytes (background activity), respectively. Background IL-13 secretion was 52.9 ± 8.9 pg per ml (mean ± SEM, n = 20), and background AC and PDE activities were 23.5 ± 4.5 pmol per min per mg protein, and 26.3 ± 5.6 pmol per min per mg protein, respectively.

epitope(s) derived from Dp may be presented on a monocyte surface in the context of MHC molecules (**Fig 7**). When the antigen/MHC complex interacts with the TCR/CD3 complex on the T cell surface, the interaction may trigger the activation of phospholipase C (PLC), which is closely linked to the TCR/CD3 complex. Though TCR/CD3 complex does not directly couple to AC, it is reported that PLC-mediated signals may indirectly activate AC (Kvanta *et al*, 1990; Bihoreau *et al*, 1991). The activated PLC hydrolyzes phosphatidyl inositol and generates IP_3 and diacylglycerol; the former mobilizes Ca^{2+} from the intracellular store, whereas the latter activates protein kinase C and induces its translocation to the membrane (**Fig 7**) (Patel *et al*, 1987; Park *et al*, 1992). It is reported that intracellular Ca^{2+} forms complex with cytosolic calmodulin and the Ca^{2+}/calmodulin complex binds to AC and activates this enzyme, whereas protein kinase C directly activates AC by phosphorylation (Iyengar, 1993). We thus tested if the Dp-induced activation of AC in AD T cells may also involve PLC-mediated signals. We preincubated AD T cells with various signal transducing enzyme inhibitors, such as PLC or protein kinase C inhibitor, and examined their inhibitory effects on Dp-induced activation of AC. When AD T cells were preincubated with the PLC inhibitor ET-18-OCH_3, the Dp-induced activation of AC was completely blocked (**Fig 8*a***), suggesting the involvement of PLC in Dp-induced activation of AC. Dp-induced activation of AC was also blocked by chelating intracellular Ca^{2+} with BAPTA/AM, indicating the involvement of Ca^{2+} signal in AC activation. The Dp-induced activation of AC was also partially blocked by calmodulin antagonist W-7 or protein kinase C inhibitor calphostin C, respectively, and the usage of both agents gave additive inhibitory effects and resulted in complete inhibition. This indicates that protein kinase C and Ca^{2+}/calmodulin may independently stimulate AC. These totally suggest that Dp-induced activation of AC may be mediated by PLC-induced two second messengers, intracellular Ca^{2+} signal and activated protein kinase C. As Ca^{2+} functions as a cofactor for protein kinase C, Ca^{2+} may be required for both protein kinase C-mediated stimulation of AC and Ca^{2+}/calmodulin-mediated stimulation of AC. This possibility is supported by the results that BAPTA/AM completely, not partially, inhibited the Dp-induced activation of AC.

We then analyzed if Dp may activate PLC in AD T cells, by measuring the level of IP_3, which is the product of PLC-mediated hydrolysis of phosphatidyl inositol. Dp increased the IP_3 level in AD T cells, indicating the activation of PLC by the Dp stimulus (**Table I**); however, Dp also increased the IP_3 level in Dp-specific Th1 cell lines from normal donors, and PPD increased the IP_3 level in AD T cells, although in these systems the antigens did not activate AC (**Fig 5*a***). PPD did not increase IP_3 level in Dp-specific Th1 cell lines from normal donors. As PLC-mediated signals are known to induce proliferation (Nishizuka, 1984), we examined the proliferative response to Dp or PPD in AD or normal T cells. The results on proliferation mostly paralleled those of IP_3; Dp enhanced

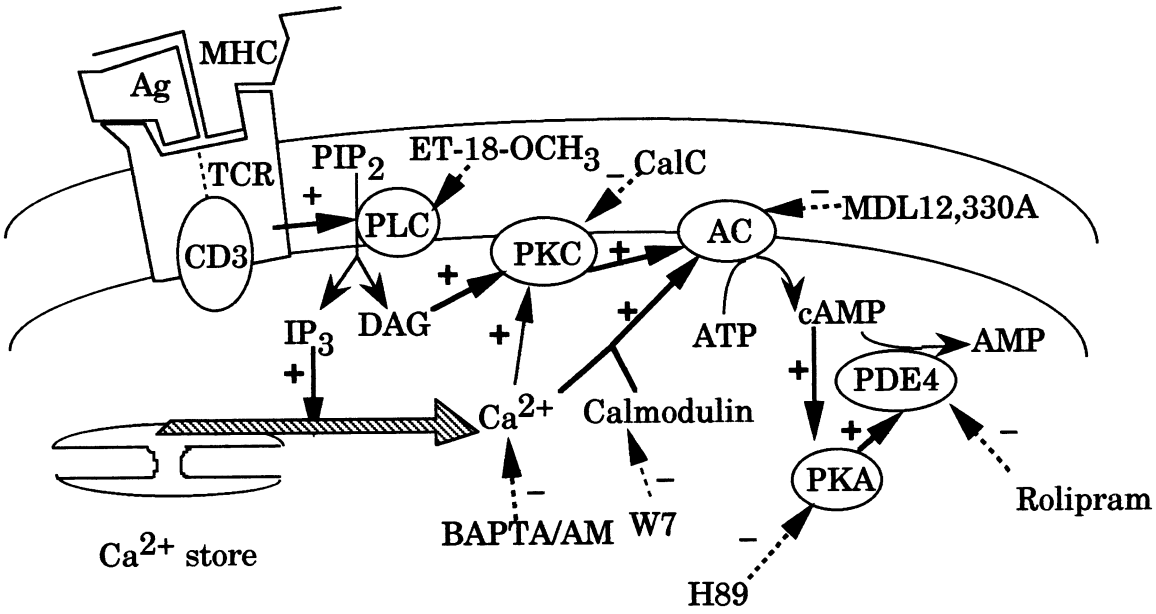

Figure 7. The schematic diagram for antigen-induced signaling cascade related to cAMP and the effects of agonists and antagonists. A solid arrow with (+) means stimulation whereas a dotted arrow with (–) means suppression, respectively. Abbreviations used: AC, adenylate cyclase; Ag, antigen; CalC, calphostin C; DAG, diacylglycerol; IP_3, inositol 1,4,5-triphosphate; MHC, major histocompatibility complex; PDE, cyclic nucleotide phosphodiesterase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; TCR, T cell receptor.

[^{3}H]thymidine uptake both in AD T cells and in Dp-specific Th1 cell lines from normal donors. PPD enhanced [^{3}H]thymidine uptake only in AD T cells, and did not in Dp-specific Th1 cell lines from normal donors. Thus the antigen-induced activation of PLC may commonly occur in Dp-stimulated AD T cells, Dp-stimulated Dp-specific Th1 cell lines from normal donors, and PPD-stimulated AD T cells; however, the antigen-induced activation of AC may only occur in Dp-stimulated AD T cells. This is possibly because the sensitivity of AC to the activation by PLC-mediated signals may be highly enhanced in the Dp-responsive T cell population from AD patients and/or another different signal which potentiates the PLC-mediated AC activation may act in such T cell population.

The Dp-induced activation of PDE was specifically blocked by rolipram, and not by 8mmIBMX or milrinone (**Fig 8*b***), suggesting that PDE4 may be specifically activated by Dp among PDE subtypes. The Dp-induced activation of PDE was completely blocked by AC inhibitor MDL 12,330A, and by protein kinase A inhibitor H-89, and was not affected by protein kinase G inhibitor Rp-8-Br-cGMPS. These totally suggest that AC and protein kinase A may be involved in the activation of PDE4 by Dp. As AC generates the cAMP signal and cAMP activates protein kinase A (**Fig 7**), it is indicated that an early cAMP signal via the Dp-induced activation of AC (**Figs 4** and **5*a***) may activate protein kinase A, and the activated protein kinase A may contribute to the activation of PDE4. This possibility is also supported by the strong correlation between the Dp-induced increase of AC activity and that of PDE in AD patients ($r_s = 0.931$, $n = 20$, $p < 0.0001$).

DISCUSSION

This study demonstrated that T cells from Dp-sensitive AD patients specifically produced IL-13 in response to Dp. The Dp stimulus (i) increased cAMP level of AD T cells by activating AC, and (ii) reduced its level by activating PDE. Both events were specific to Dp-sensitive AD patients, and were required for IL-13 induction by Dp. Dp-induced IL-13 secretion strongly correlated with the Dp-specific IgE value, suggesting that Dp-induced IL-13 production may contribute to the synthesis of Dp-specific IgE antibody. Though it is known that IL-4 enhances whereas IFN-γ suppresses IgE synthesis of B cells (Punnonen and de Vries, 1994), IL-4 and IFN-γ responses to Dp in AD patients were very low and were not different from those in normal donors at protein and mRNA levels. This indicates that neither IL-4 nor IFN-γ may be involved in the production of Dp-specific IgE. The IFN-γ response to Dp, however, may be latently reduced in AD T cells relative to normal T cells; the short-term culture generated Th1 cell lines showing an IFN-γ response to Dp in normal donors whereas it could not in AD patients. Thus the impairment of an IFN-γ response to Dp may also be related to the increased production of Dp-specific IgE antibody in AD patients. We are now trying to establish Dp-specific T cell clones from AD patients, and to examine their cytokine profile and ability to enhance Dp-specific IgE synthesis of autologous B cells *in vitro*.

The early rise of cAMP ($\leqslant 5$ min) after the Dp stimulus was required for IL-13 production and thus seems to be at least one of the triggering signals for IL-13 induction. As cAMP activates protein kinase A, this kinase may upregulate the early step of IL-13 production. One possible mechanism for the upregulation is that protein kinase A may phosphorylate certain transcription factors involved in IL-13 gene expression and thus promote their activity. It is reported that protein kinase A enhances the transcriptional stimulatory activity of activator protein-2 (AP-2) on several genes containing the AP-2 element (Gao *et al*, 1997; Garcia *et al*, 1999); the AP-2 element is also present in the 5′-flanking region of IL-13 genes (Smirnov *et al*, 1995). Other cAMP-related transcription factors, such as cAMP-responsive element-binding protein (CREB) or activation transcriptional factor-1 (ATF-1), may also be involved in IL-13 gene expression, although their roles in IL-13 transcription have not been identified.

The previous studies suggest the cross-talk interaction between TCR/CD3-mediated signals and cAMP (Bihoreau *et al*, 1991). It is reported that stimulation of TCR/CD3 by anti-CD3 increased cAMP levels in human Jurkat T cells (Kvanta *et al*, 1990; Bihoreau *et al*, 1991). In this system, the stimulation of TCR/CD3 activated PLC, and the PLC-induced second messengers, protein kinase C activation and/or intracellular Ca^{2+} signal appeared to activate AC (**Fig 7**) (Bihoreau *et al*, 1991; Iyengar, 1993). To date, eight different isotypes of AC have been isolated, and these different types are localized in a tissue-type-specific manner and a cell-type-specific manner (Iyengar, 1993). Protein kinase C stimulates AC types 2 and 7, and Ca^{2+}/calmodulin stimulates AC types 1 and 3,

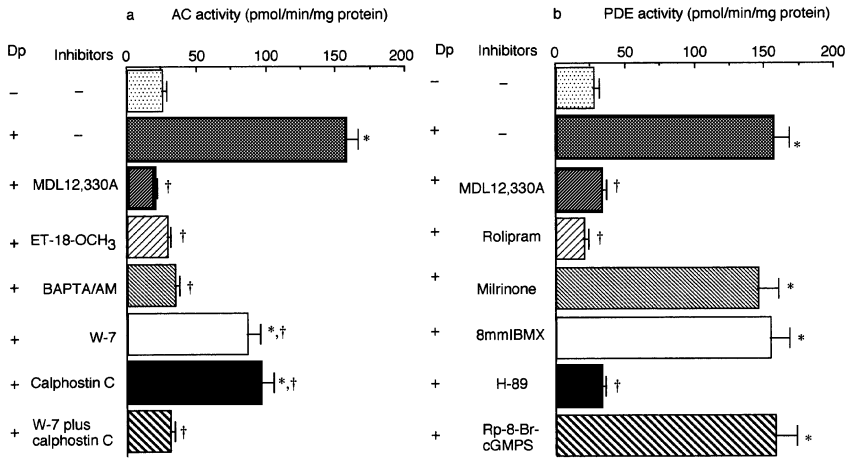

Figure 8. Signal transducing enzyme inhibitors' effects on Dp-induced activation of AC and PDE in T cells from AD patients. (*a*) AC; (*b*) PDE. T cells from AD patients were preincubated for 30 min with medium alone or with medium containing signal transducing enzyme inhibitors, washed, and then added to autologous Dp-pulsed or nonpulsed monocytes. AC activity of T cells was analyzed at 5 min and that of PDE was analyzed at 15 min of the culture, respectively. The inhibitors were MDL 12,330A (5 μM), ET-18-OCH_3 (1 μM), BAPTA/AM (10 μM), W-7 (50 μM), calphostin C (0.1 μM), rolipram (5 μM), milrinone (10 μM), 8mmIBMX (10 μM), H-89 (0.1 μM), and Rp-8-Br-cAMPS (10 μM). The data are mean ± SEM (n = 7). *$p < 0.05$ *versus* control cultures with Dp-nonpulsed monocytes. †$p < 0.05$ *versus* cultures with Dp-pulsed monocytes without inhibitor preincubation.

although Ca^{2+} directly inhibits AC types 5 and 6 without involving calmodulin (Iyengar, 1993). AC type 1 or 3 has not been detected in human T cells; however, several reports support that AC in human T cells is activated by Ca^{2+}/calmodulin (Kvanta *et al*, 1990; Bihoreau *et al*, 1991). In our study, Dp activated PLC and induced the proliferative response both in AD T cells and in Dp-specific Th1 cell lines from normal donors; however, Dp activated AC only in AD T cells showing an IL-13 response to Dp, and did not in the nonatopic Th1 cell lines showing an IFN-γ response to Dp. It is thus possible that the Dp-induced activation of AC may favor the IL-13 production over IFN-γ, and this possibility should further be examined. Presumably, the AC of the Dp-responsive T cell population from AD patients may be more susceptible to the activation by protein kinase C and/or Ca^{2+}/calmodulin, indicating the abundance of AC types 2 and 7 and/or 1 and 3. On the other hand, the AC of Dp-specific Th1 cell lines from normal donors may be less sensitive to such activation and/or may be inhibited by the Ca^{2+} signal, indicating the low proportion of AC types 2 and 7 or 1 and 3 and/or the contribution of AC types 5 and 6. Further studies should elucidate the possible difference in AC isotype by generating Dp-specific T cell clones from AD patients and normal donors and clarifying the AC isotype of each clone. Another possible difference is that the culture of T cells and monocytes from AD patients may contain higher amounts of endogenous AC agonists, such as prostaglandins, as compared with the culture from normal donors; such AC agonists also may facilitate the activation of AC by PLC-mediated signals. It is reported that the prostaglandin E_2 production of monocytes is markedly enhanced in AD patients compared with normal donors (Chan *et al*, 1993). Bihoreau *et al* (1991) also reported that cAMP increase by anti-CD3 antibody alone was only slight in magnitude but was markedly potentiated by the combination with AC agonists such as prostaglandin E_2, cholera toxin, or forskolin. As opposed to Dp, Th1-antigen PPD did not induce the activation of AC in AD T cells. Possibly, Dp-responsive T cells from AD patients may be a distinct population among whole T cells, and may have the AC isotype, which is much more sensitive to activation by PLC-mediated signals than that of the PPD-responsive T cell population. This possibility should further be investigated by establishing Dp- and PPD-specific T cell clones from AD patients.

The activation of PDE by Dp occurred after that of AC in AD T cells. Among various PDE isotypes, only PDE4 appeared to be activated by Dp, and was required for Dp-induced IL-13 production. The Dp-induced activation of PDE was inhibited by protein kinase A inhibitor, H-89 and AC inhibitor MDL 12,330A. These results indicate the involvement of AC and protein kinase A in the Dp-induced activation of PDE4. It is also reported that protein kinase A promotes the activity of PDE4D3 by phosphorylation at serine[54] (Sette and Conti, 1996). Possibly, Dp may initially activate AC and generate cAMP signals, which may promote protein kinase A activity, and this kinase may thus phosphorylate and activate PDE4 (**Fig 7**). Then the activated PDE4 may hydrolyze cAMP and thus reverse the cAMP amount and protein kinase A activity to basal level. This appears the negative feedback control by cAMP-related signaling pathways. PDE4 inhibitor, rolipram retained the elevated cAMP level after Dp stimulus, and this led to the suppression of IL-13 production. It is thus indicated that the prolonged accumulation of cAMP may downregulate IL-13 production. Possibly, protein kinase A may have a dual function; it may promote the early step of IL-13 production whereas in the later phase, it may act negatively. One possible mechanism for the inhibition is that protein kinase A may promote the degradation of IL-13 mRNA. It is also reported that cAMP-elevating agents decrease the stability of IL-4 mRNA in anti-CD3/anti-CD28-activated human T cells (Borger *et al*, 1996). Another possible mechanism is that protein kinase A may suppress the activity of PLC, which is initially promoted by the antigen stimulation of TCR (**Fig 7**). It is reported that protein kinase A phosphorylates PLC-γ1 at serine[1248] and thus interferes with the tyrosine phosphorylation of PLC-γ1, and this reduces the activity of this enzyme (Park *et al*, 1992). The inhibition by cAMP may also occur at post-translational level; it is reported that the accumulation of cAMP inhibits the assembly of microtubules in the Golgi/trans-Golgi area, and thus interrupts the intracellular transport and secretion of proteins (Lichtenstein and Henney, 1974; Bennet *et al*, 1984), and this may be the case for IL-13.

Dp- or PPD-induced IFN-γ production was not blocked by the AC inhibitor and thus may not require the activation of AC and the resultant cAMP signal. Beside the cAMP-elevating agent, rolipram inhibited the Dp- or PPD-induced IFN-γ production. These suggest that cAMP may only inhibit IFN-γ production without stimulatory effects. Recent studies suggest that the inhibition may occur mainly at transcriptional level; the proximal element of the IFN-γ promoter contains binding sites for AP-1 and for CREB/ATF-1, which are overlapping (Aune *et al*, 1997). When protein kinase A is activated by cAMP, it phosphorylates CREB and/or ATF-1, and thus enhances their binding to the IFN-γ promoter. The binding of CREB and/or ATF-1 to the IFN-γ promoter competitively inhibits the binding of AP-1, and thus represses the transcriptional activity of the promoter (Penix *et al*, 1996; Zhang *et al*, 1998). Such competitive inhibition by CREB/ATF-1 does not occur at the AP-1 binding site on the IL-2 or IL-4 promoter and thus may be specific to the IFN-γ promoter (Zhang *et al*, 1998). The inhibitory effects of cAMP on IFN-γ production may also occur at the pretranscriptional or post-transcriptional level, some of which may be common to those on IL-13 production as described above.

In this study, PDE4 and AC inhibitors suppressed Dp-induced IL-13 production of AD T cells. This indicates that these agents may also *in vivo* suppress the Dp-specific IgE synthesis via the

inhibition of IL-13 production and thus prevent the development of AD. In particular, the AC inhibitor blocked Dp-induced IL-13 production without reducing that of IFN-γ. To date, the therapeutic efficacy of the AC inhibitor has not been reported and thus the drug may provide a new insight into the treatment of AD patients. We are now studying if the AC or PDE4 inhibitor may *in vitro* suppress the Dp-specific IgE synthesis in peripheral blood mononuclear cells from AD patients.

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