

Evaluation of mite allergen-induced Th1 and Th2 cytokine secretion of peripheral blood mononuclear cells from atopic dermatitis patients: association between IL-13 and mite-specific IgE levels

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Abstract

There have been several reports about Th1/Th2 imbalances in atopic dermatitis (AD), but there have been few precise investigations about the differences between Th1 and Th2 cytokine secretion patterns of peripheral blood mononuclear cells (PBMCs) in such patients. We cultured PBMCs, taken from AD patients and healthy subjects, with dust mite extract (DME) and measured subsequent immunoreactive interferon (IFN)- γ (Th1 cytokine), interleukin (IL)-4, IL-5, and IL-13 (Th2 cytokines) levels in the supernatants by ELISA assays. There is a difference between IL-4 and IL-13 secretion patterns by DME-stimulated PBMCs in AD subjects; immunoreactive IL-4 levels were detectable maximally within 24-h cultures, while IL-13 levels increased time-dependently within 7-day cultures. IL-13 levels were significantly elevated in AD subjects compared to healthy subjects, while IFN- γ levels did not significantly differ between the two groups. IL-13 levels were significantly higher in AD patients who had high levels (> 100 U/ml) of Dermatophagoides pteronyssinus-specific IgE (Dp-IgE) than in those AD patients who had low levels (< 10 U/ml) of Dp-IgE. Tacrolimus (FK-506), at a concentration of 10^{-8} M, significantly inhibited DME-induced IL-13 production from PBMCs. These findings suggest that IL-13 produced by Th2 cells are involved in IgE overproduction in AD subjects. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Atopic dermatitis; Mononuclear leukocytes; Th2 cells; Interleukin-4; Interleukin-13; Tacrolimus

1. Introduction

The cause of atopic dermatitis (AD) is multifactorial. Immunological abnormalities as well as physiological and biochemical defects of the skin barrier structure are involved in the pathogenesis. Furthermore, genetic and environmental factors can modify these abnormalities [1].

Abbreviations: AD, atopic dermatitis; DME, dust mite extract; Dp, dermatophagoides pteronyssinus; IFN, interferon; IL, interleukin; PBMC, peripheral blood mononuclear cell.

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Dust mite is one of the major allergens in AD, especially in adult type of AD [2]. The frequency of mite allergen hypersensitivity is variously reported; these differences may be derived from regional differences and/or generational differences. In Japan, the positive rate of mite-specific IgE (RAST) is about 70% in adult type of AD patients [3]. We have already reported that 2/3 of adult type AD patients with high mite-specific IgE show positive reactions to skin patch test with 1% of crude mite extract while none of healthy controls shows positive reactions to the allergen [4]. Furthermore, other researchers have reported that AD lesions are cleared by avoiding mite allergen in some cases [5–7]. These findings suggest that dust mite can be involved in the pathogenesis of AD.

As an immunological abnormality, there are several reports that suggest a Th1/Th2 imbalance (i.e. an uneven proportion of Th1 and Th2 immune cells) in AD. It has also been reported that there are many mite allergen-specific T cells in AD lesions, and most of these are interleukin (IL)-4 producing T cells [8]. Furthermore, another report has shown that mononuclear cells collected from AD lesions produce greater amounts of IL-4 and IL-5 compared with either psoriasis vulgaris lesions or healthy skin [9]. This report has also noted that the IL-4/interferon (IFN)- γ ratio is significantly increased in AD lesions [9]. These findings suggest that AD lesions are Th2 predominant.

There have also been many reports about the predominance of Th2 cells in peripheral blood samples of AD patients. Peripheral blood mononuclear cells (PBMCs) from AD subjects produce large amounts of IL-4 and small amounts of IFN- γ compared with those from healthy subjects, as elicited by allergen stimulation [10]. PBMCs from AD subjects with high serum IgE levels spontaneously produce IgE *in vitro*, and this IgE production is further augmented by IL-4 stimulation [11]. These findings suggest that AD is a Th2-favoring disease that results in high IgE levels in sera.

On the other hand, some findings suggest that Th1 cytokine production is important in AD. Grewe et al. have reported that IFN- γ mRNA is

augmented in 13 of 15 cases, while IL-4 mRNA is augmented in 4 of 15 cases and concluded that AD lesions are Th1 predominant [12]. Furthermore, they have performed patch tests using mite-allergen and have investigated the chronological cytokine profiles of induced lesions. This investigation shows that IL-4 mRNA is augmented at 24 h post-allergen exposure, while IL-4 mRNA is decreased and IFN- γ and IL-12 p35 mRNA are augmented at 48 h [13]. They have concluded that chronic lesions of AD would undergo shifts in PBMC population profiles from Th2 to Th1 by IL-12 augmentation. These data suggest that Th1 cytokines would be predominant in certain conditions of AD.

However, it is still unclear to what extent Th1 and Th2 cytokines are produced in AD and how imbalances in Th1/Th2 cell populations are involved in the pathogenesis of AD. In this study, we cultured PBMCs, taken from AD and healthy subjects, with dust mite allergen, and measured immunoreactive IFN- γ , IL-4, IL-5, and IL-13 levels in the supernatants by ELISA assay. Comparisons were made between each cytokine level and the AD subject's IgE level. Furthermore, we examined the regulation of IL-13 production by tacrolimus (FK-506), which is an effective immunosuppressant and has been used recently in treating AD skin lesions [14].

2. Materials and methods

2.1. Subjects

Thirty-four Japanese patients afflicted with AD (18 males and 16 females, from 18 to 41 years of age), who fulfilled the criteria of AD by Hanifin and Rajka [15] and who had not suffered from any other active allergic diseases such as asthma, were enrolled in this study. Eight healthy subjects (seven males and one female, from 21 to 35 years of age) who have not experienced allergic diseases and have low levels (< 10 U/ml) of *Dermatophagoides pteronyssinus* (Dp)-specific IgE levels (Dp-IgE) were enrolled as controls.

2.2. Culture of PBMCs with DME

Dust mite crude extract (DME) was purchased from LSL Co. Ltd. (Tokyo, Japan). The DME was extracted by whole mite body of *Dp* and *Dermatophagoides farinae* (*Df*) equally and its protein concentration was 5 mg. It was diluted in 1 ml of water and stored at -80°C until use.

First, for a preliminary study, PBMCs were taken from 3 AD and 1 healthy subjects and regulated to 2×10^6 cells/ml by RPMI1640 (Nikken biology and medical research center, Kyoto, Japan). They were cultured with 1 $\mu\text{g}/\text{ml}$ of DME in 6-well plates at 37°C for 12 h, 24 h, 3 days (72 h) and 7 days (168 h).

Next, PBMCs were taken from 34 AD and eight healthy subjects and cultured with or without 1 $\mu\text{g}/\text{ml}$ of DME at 37°C for 7 days. Finally, in order to investigate the effects of tacrolimus for cytokine production by PBMCs, PBMCs were taken from 5 AD subjects and cultured with or without 1 $\mu\text{g}/\text{ml}$ of DME in 6-well plates, and 10^{-8} to 10^{-5} M concentrations of tacrolimus (Fujisawa Pharmaceutical Co. Ltd. Tokyo, Japan) or 10^{-8} M concentrations of dexamethasone (Calbiochem-Novabiochem Co. La Jolla, CA) at 37°C for 3 days.

It was confirmed that more than 80% of the cells were viable after the incubation by using the Tripzan blue dye exclusion test. All supernatants were collected and stored at -80°C until use.

2.3. Measurement of cytokine concentration by ELISA

Immunoreactive IL-4 and IL-13 levels from PBMCs were quantified with human IL-4 and IL-13 ELISA kits (Diaclone Res. Besancon, France), and immunoreactive IL-5 and IFN- γ levels were quantified with human IL-5 and IFN- γ ELISA kits (R&D systems, Minneapolis, MN). These assays employed the quantitative sandwich enzyme immunoassay technique. Optical density was measured at 450 nm with a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories Inc. Hercules, CA). The cytokine concentration was estimated from the standard curve generated by a curve-fitting program. The minimum detectable

IL-4, IL-13, IL-5, and IFN- γ concentrations were 0.5, 1.5, 3.0, and 3.0 pg/ml, respectively.

2.4. Statistical analysis

Immunoreactive cytokine levels were expressed as mean values $\pm$ SD. The statistical analyses were performed by the Student's *t* test for comparing the cytokine levels among different clinical groups (such as AD vs. healthy). In turn, the Pearson's correlation coefficient test was utilized to determine if there were any correlations among cytokine levels. Differences were defined as statistically significant at $P < 0.05$.

3. Results

3.1. Chronological differences in IL-4, IL-5, IL-13, and IFN- γ levels secreted by PBMCs cultured with DME

PBMCs taken from 3 AD and 1 healthy subjects were cultured with 1 $\mu\text{g}/\text{ml}$ concentration of DME at 37°C for 12 h, 24 h, 3 days (= 72 h), and 7 days (= 168 h) and immunoreactive IL-4, IL-5, IL-13, and IFN- γ levels in culture supernatants were measured (Fig. 1a–h).

Immunoreactive IL-4, IL-5, IL-13, and IFN- γ levels were extremely low in culture supernatants without DME in both AD and healthy subjects (Fig. 1b, d, f, h).

AD patients demonstrated IL-4 levels that were detectable maximally within 24 h, and which progressively decreased in the day 3 and day 7 cultures (Fig. 1a). The cytokine kinetics curve for IL-5, IL-13 and IFN- γ is different in that a peak was not reached until day 7; thus, the concentration increased in a time-dependent fashion for the duration of the experiment (Fig. 1c, e, g).

3.2. Differences in IL-5, IL-13, and IFN- γ levels secreted by PBMCs from AD and healthy subjects cultured with DME

PBMCs taken from 34 AD and eight healthy subjects were cultured with or without 1 $\mu\text{g}/\text{ml}$ of DME at 37°C for 7 days, and differences in

immunoreactive IL-4, IL-5, IL-13, and IFN- γ levels were examined (Fig. 2a–f).

Immunoreactive IL-5 levels produced by PBMCs from healthy subjects cultured with DME

were significantly higher than those without DME ($P < 0.001$) (Fig. 2b). Immunoreactive IL-5 levels produced by PBMCs from AD subjects cultured with DME were also significantly higher than

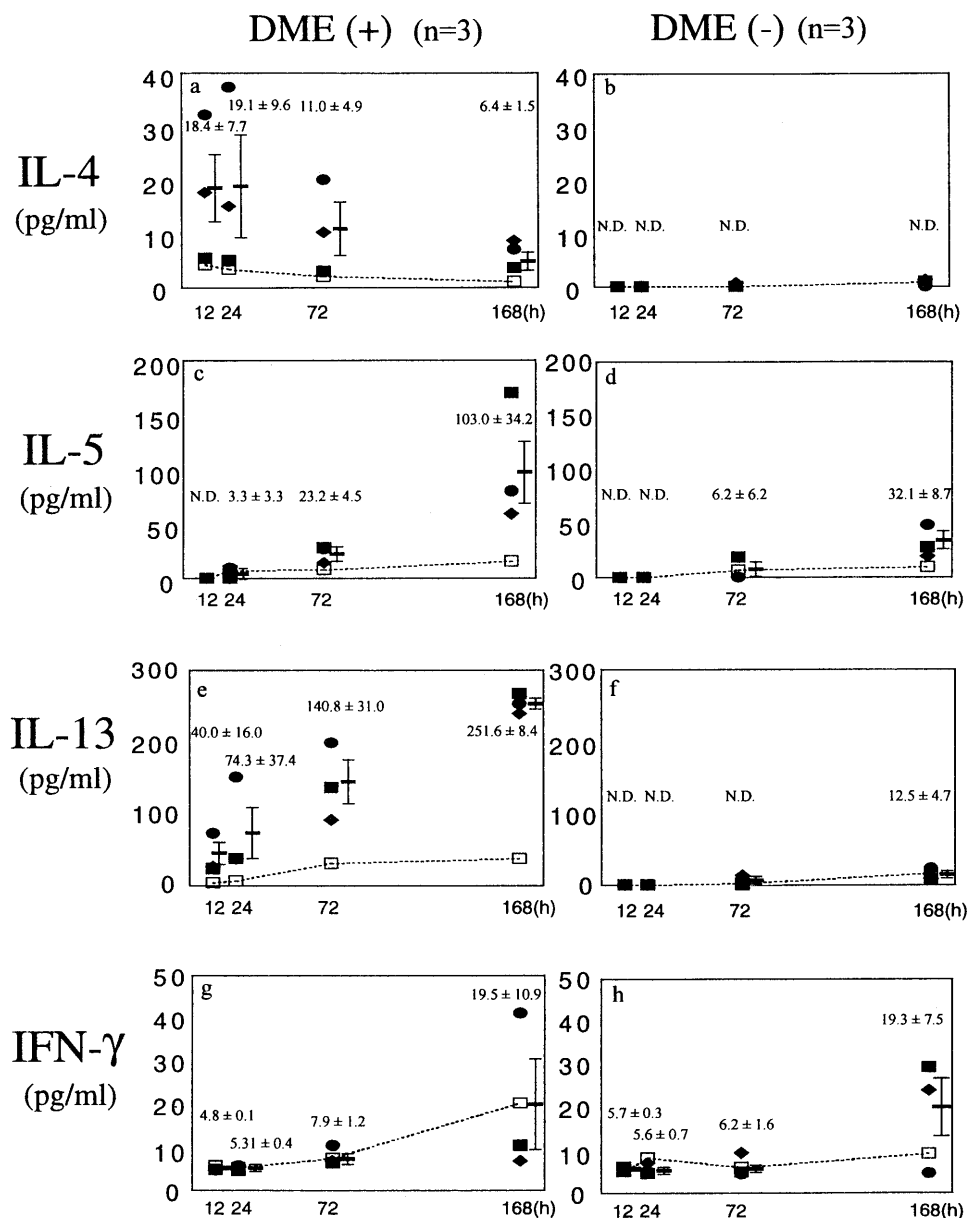


Fig. 1. Chronological differences in IL-4, IL-5, IL-13 and IFN- γ levels secreted by PBMCs cultured with DME. In AD subjects, immunoreactive IL-4 levels were detectable maximally within 24 h, while the cytokine kinetics curve for IL-5, IL-13 and IFN- γ is different in that a peak was not reached until day 7 (a, c, e, g). All cytokines were extremely low in culture supernatants without DME in both AD and healthy subjects (b, d, f, h). (■●◆: AD subjects ($n = 3$), □: healthy subject).

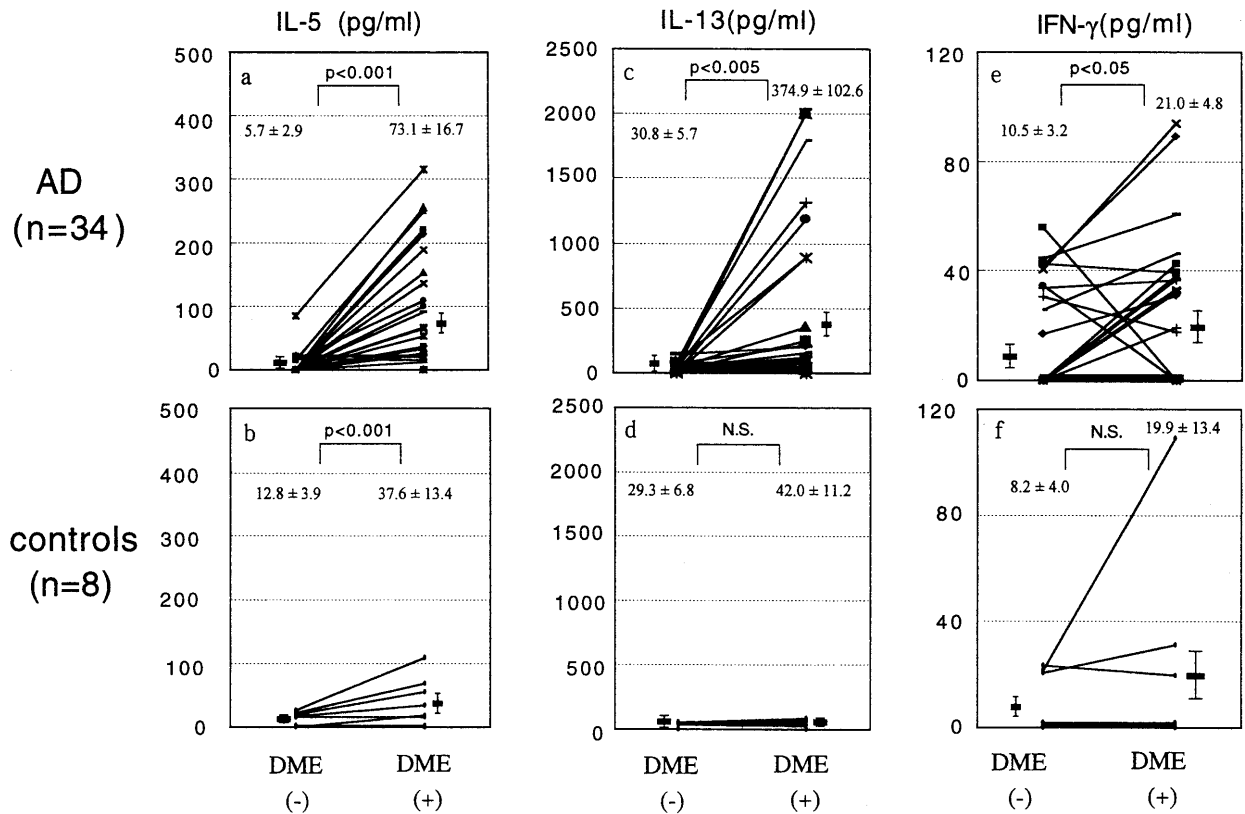


Fig. 2. Differences in IL-5, IL-13 and IFN- γ levels secreted by PBMCs from AD and healthy subjects cultured with DME. In AD subjects, immunoreactive IL-5 and IL-13 levels produced by PBMCs cultured with DME were significantly higher than those without DME ($P < 0.001$ and $P < 0.005$, respectively) (a)(c). Individual IFN- γ levels with DME was higher than those without DME in 14 of 34 cases, but were lower in four of 34 cases (e). Immunoreactive IL-5, IL-13 and IFN- γ levels were not significantly augmented by DME exposure (b)(d)(f).

those without DME ($P < 0.001$) (Fig. 2a). There was no significant differences between AD and healthy subjects in IL-5 secretion without DME. There was, in fact, a difference, as the IL-5 levels by PBMCs from AD subjects with DME were nearly two-fold higher than those from healthy subjects, but this difference did not reach the threshold of statistical significance.

Immunoreactive IL-13 levels produced by PBMCs from healthy subjects cultured with DME were higher than those without DME, but this difference was not statistically significant (Fig. 2d). In contrast, the IL-13 levels by PBMCs from AD subjects cultured with DME were 12-fold higher than those without DME and this difference was statistically significant ($P < 0.005$) (Fig.

2c). Though no significant difference was observed between AD and healthy subjects in IL-13 levels without DME, IL-13 levels produced by PBMCs from AD subjects cultured with DME were significantly higher than those from healthy subjects ($P < 0.005$).

In evaluating the IFN- γ levels by PBMCs from healthy subjects, no significant difference was observed between PBMCs cultured with and without DME (Fig. 2f). In AD subjects, individual IFN- γ levels were quite variable: the levels in culture supernatants with DME were higher than those without DME in 14 of 34 cases, but were lower in 4 of 34 cases, and no appreciable differences were observed between the remaining cases. On average, IFN- γ levels from AD subjects with DME

were significantly higher than those without DME ($P < 0.05$) (Fig. 2e). There was not a significant difference between AD and healthy subjects in IFN- γ levels produced by the PBMCs, irrespective of the presence or absence of DME.

Immunoreactive IL-4 levels were under 10 pg/ml in all AD and healthy subjects (figure was not shown).

3.3. Correlation among DME-induced cytokine levels

Correlations were made among various immunoreactive cytokine levels produced by PBMCs from AD subjects. These PBMCs were cultured with 1 μ g/ml of DME. A significant positive correlation was demonstrated between IL-5 and IL-13 levels (Fig. 3a). IFN- γ levels, on the other hand, were negatively and significantly correlated with IL-5 and IL-13 levels (Fig. 3b, c).

3.4. Comparison between DME-induced cytokine level and the patient's Dp-IgE level

AD subjects were divided into three groups according to their Dp-IgE levels as follows: low Dp-IgE (< 10 U/ml), moderate Dp-IgE (10–100 U/ml), and high Dp-IgE group (> 100 U/ml). The number of patients in each group was 13, six and 15, respectively. Comparisons were made among Dp-IgE groups and their immunoreactive

cytokine levels in culture supernatants with 1 μ g/ml of DME exposure (Table 1).

Immunoreactive IL-5 levels in the high Dp-IgE group were significantly higher than those in the low Dp-IgE group ($P < 0.005$). IL-13 levels in the high Dp-IgE group were also significantly higher than those in the low Dp-IgE group ($P < 0.005$).

Immunoreactive IFN- γ levels were augmented by DME-stimulation in seven of 13 cases in the low Dp-IgE group. In the moderate Dp-IgE group, IFN- γ levels were augmented by DME-stimulation in one of six cases, while two of six were suppressed. In the high Dp-IgE group, IFN- γ levels were augmented by DME stimulation in five of 13 cases, while they were substantially suppressed in two of 13 cases. IFN- γ levels in the high Dp-IgE group (with DME) were lower than those in the low Dp-IgE group, but the difference was not statistically significant.

No correlation was observed between these cytokines production and the eruption types, such as acute or chronic.

3.5. Tacrolimus (10^{-8} – 10^{-5} M) inhibited DME-induced cytokine production by PBMCs from AD subjects

PBMCs from 5 AD subjects were cultured with 1 μ g/ml of DME and tacrolimus at 10^{-8} – 10^{-5} M or dexamethasone at 10^{-8} M for 3 days (Fig. 4a–c).

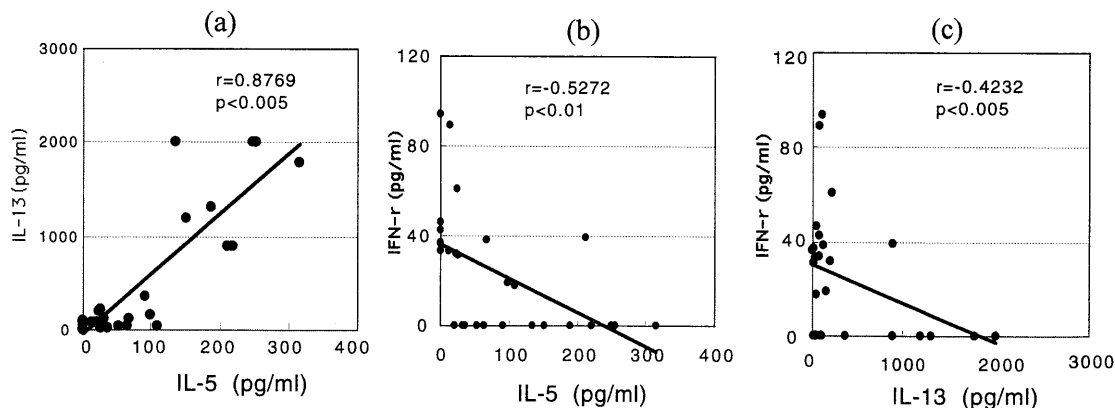


Fig. 3. Correlation among DME-induced cytokine levels by PBMCs from AD subjects. A significant positive correlation was demonstrated between IL-5 and IL-13 levels. IFN- γ levels were negatively and significantly correlated with IL-5 and IL-13 (a–c).

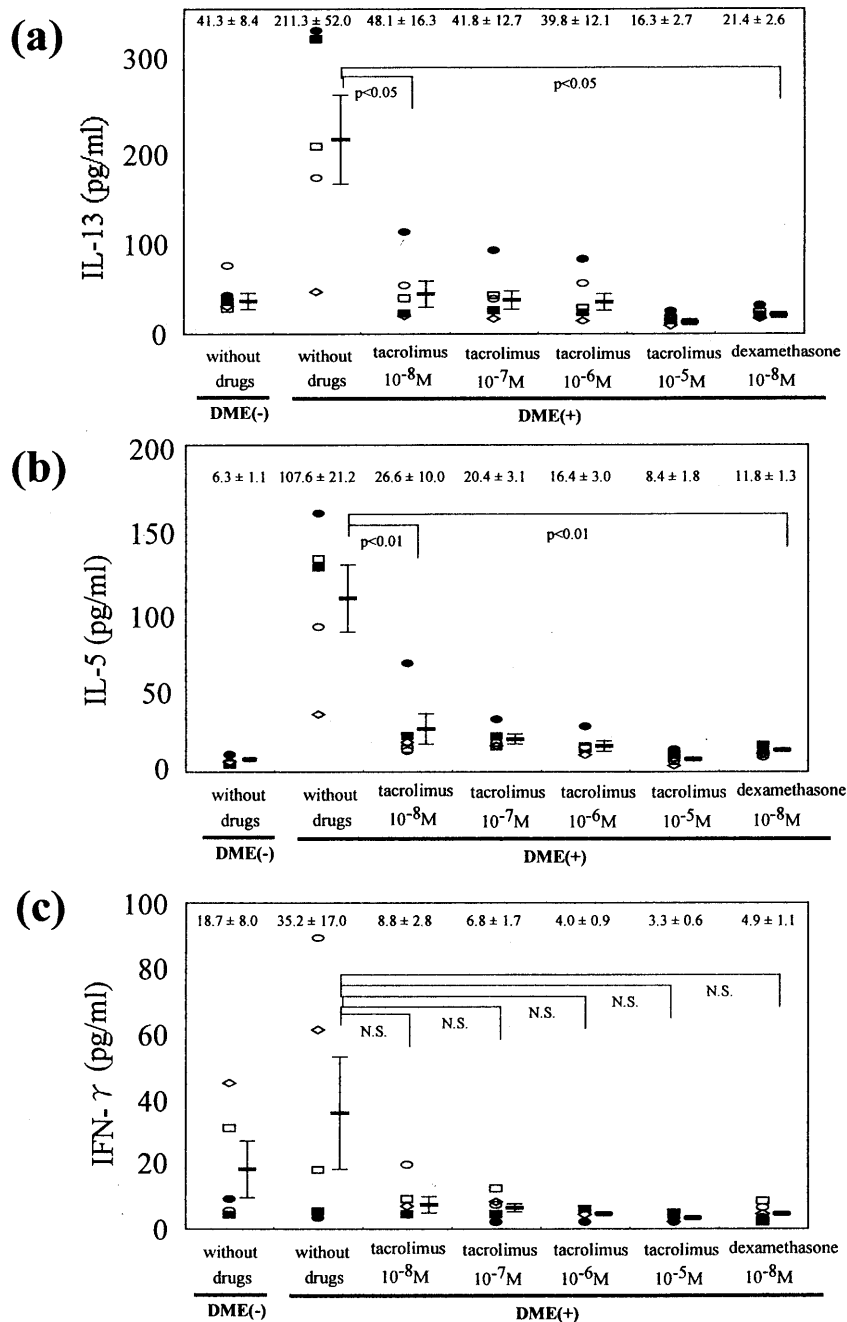


Fig. 4. Tacrolimus (10^{-8} – 10^{-5} M) inhibited DME-induced IL-13, IL-5 and IFN- γ production by PBMCs from AD subjects. Tacrolimus at 10^{-8} M significantly inhibited DME-induced IL-13 and IL-5 production by PBMCs from AD subjects (a)(b). Tacrolimus also inhibited, though not significant, DME-induced IFN- γ production. Immunoreactive IFN- γ levels with DME and 10^{-8} M of tacrolimus was as substantial as the levels of spontaneous production without DME (c).

Table 1

Comparison between dust mite extract (DME)-induced cytokine level and the patient's Dp-specific IgE (Dp-IgE) level

		AD patients			
Cytokines		Healthy controls (<i>n</i> = 8)	Dp-IgE low group <10U/ml (<i>n</i> = 13)	Dp-IgE moderate group 10–100U/ml (<i>n</i> = 6)	Dp-IgE high group >100U/ml (<i>n</i> = 15)
IL-5 (pg/ml)	DME (–)	12.8 ± 3.9	3.1 ± 2.1	2.5 ± 2.5	9.4 ± 6.6
	DME (+)	37.6 ± 13.4	7.1 ± 3.1	79.3 ± 30.4	131.2 ± 29.4
			$P < 0.005$		
IL-13 (pg/ml)	DME (–)	29.3 ± 6.8	28.3 ± 8.1	27.9 ± 13.8	34.1 ± 9.8
	DME (+)	42.0 ± 11.2	49.7 ± 17.0	179.4 ± 141.0	735.0 ± 190.4
			$P < 0.005$		
IFN- γ (pg/ml)	DME (–)	8.2 ± 5.7	11.3 ± 5.2	16.5 ± 7.7	7.0 ± 4.9
	DME (+)	19.9 ± 13.4	32.2 ± 10.0	17.2 ± 8.6	12.3 ± 4.7

IL-13 and IL-5 production induced by DME were significantly inhibited by 10^{-8} M concentrations of tacrolimus ($P < 0.05$ and $P < 0.01$, respectively). The inhibition of IL-13 and IL-5 production by 10^{-5} M of tacrolimus were as substantial as that inhibited by dexamethasone at a concentration of 10^{-8} M. Tacrolimus also inhibited IFN- γ production at 10^{-8} M, but as the amount of mite-induced IFN- γ production was originally a little, this inhibition was not statistically significant.

4. Discussion

In this study, we cultured PBMCs from AD subjects with or without DME and measured immunoreactive cytokine levels in the supernatants. The data obtained from the AD subjects was compared with the data in healthy subjects. For more detailed analysis, AD subjects were divided into three groups according to their Dp-IgE levels, before comparing their immunoreactive cytokine levels. Furthermore, we examined the regulation of IL-13 production by tacrolimus and found the following: (1) there is a difference between IL-4 and IL-13 secretion patterns by 1 μ g/ml DME stimulation of PBMCs from AD subjects; IL-4 levels were detectable maximally within the 24 h culture, while IL-13 levels increased in a time-dependent fashion within the 7-day culture; (2) IL-13 levels were significantly

augmented in AD subjects compared with healthy subjects, while IFN- γ levels showed no significant difference between both groups; (3) IL-13 levels of AD subjects with high levels of Dp-IgE were significantly higher than those with low levels of Dp-IgE; and (4) 10^{-8} M of tacrolimus significantly inhibited DME-induced IL-13, IL-5 and IFN- γ production by PBMCs.

Previous researches have indicated that IL-4 plays a primary role in the overproduction of IgE in allergic diseases such as AD [16], although IL-13 is also shown to induce IgE synthesis. IL-13 is mainly produced by Th2 cells, though Th0, CD8 positive cells and dendritic cells also produce IL-13 [17]. IL-13 shares many biologic activities with IL-4, which is due to the fact that the IL-13- and IL-4-receptor complexes share the IL-4-receptor alpha-chain that is important for signal transduction [18]. IL-13 is related to IgE and IgG₄ synthesis by B cells [19]. Although its effects are slightly weaker than those produced by IL-4, IL-13 produces IgE independently by stimulating B cells [19].

There have been several reports concerning the overexpression of IL-4 and IL-13 in AD. It is shown by in situ hybridization methods that IL-13 mRNA expression is augmented in both the lesional and non-lesional skin of AD patients, while IL-4 mRNA is augmented only in acute lesions of AD [20]. Another study has shown that PBMCs obtained from AD subjects do not express IL-4 mRNA in non-stimulated conditions,

while IL-13 mRNA expression is augmented compared with that seen in psoriasis patients and healthy subjects [21]. These findings suggest that IL-13 is important in the pathogenesis of AD.

In this study, a difference between IL-4 and IL-13 secretion patterns was demonstrated by DME-stimulated PBMCs from AD subjects. Specifically, IL-4 secretion was spontaneous, while IL-13 secretion was continuous. This result also support the findings by de Waal Malefyt et al. that IL-4 mRNA expression by Th2-like cell clone NP-12 is only detected within 4 h after the cells are cultured with PMA, while IL-13 mRNA expression is detected still after 24 h of activation [17]. Moreover, our experiments also demonstrated that immunoreactive IL-13 levels were much higher than immunoreactive IL-4 levels. IL-13 levels were high especially in culture supernatants of PBMCs obtained from AD subjects with high Dp-IgE levels. These data suggest that IL-13 is related to the overproduction of IgE, although IL-13 is noted to have slightly weaker effects than those induced by IL-4 [19]. It can be speculated that IL-4 works only the early stage in allergic inflammation after DME exposure.

In this study we also noted IFN- γ secretion by PBMCs from AD subjects and found that immunoreactive IFN- γ levels of the low Dp-IgE group tended to be higher than those in the high Dp-IgE group. In contrast, immunoreactive IL-13 levels from the low Dp-IgE group were much lower than those of the high Dp-IgE group. These results collectively suggest that IFN- γ production by Th1 cells suppresses Th2 cytokines such as IL-13, resulting in inhibition of IgE production. It is well established that many adult AD subjects have abnormal serum IgE levels, and most of them have high levels of Dp-IgE. In this study, 15 of 34 AD subjects have quite high levels of Dp-IgE (> 100 U/ml). Our data suggest that low IgE levels in some AD subjects is related to IFN- γ production by Th1 cells. In other words, the Th1/Th2 population balance is important for preventing IgE overproduction in the low Dp-IgE group of AD.

These experiments also showed that 10^{-8} M of tacrolimus markedly suppressed IL-13 and IL-5 production by PBMCs cultured with DME. Clinically

such results generate interest because tacrolimus is an effective immunosuppressant that is used in solid organ transplantation [22]. Recently, topical application of tacrolimus has been shown to be effective in treating the skin lesions of AD subjects [14]. This drug blocks calcineurin activation, which is important for proper intracellular T cell signaling [23]. Our study suggests that some anti-inflammatory effects of tacrolimus occur by blocking cytokine production from T cells and provides conclusive evidence that this drug has strong anti-inflammatory effects and is useful for treating inflammatory diseases such as AD.

By the way, an intriguing finding has been reported by Nagai et al., that tacrolimus potentiates the Th2 cell-mediated IgE antibody production in vivo [24]. This finding seems to conflict with our result of inhibitory effect of tacrolimus on Th2 cytokines production. They have demonstrated that tacrolimus augments IgE production in mouse when dinitrofluorobenzene (DNFB) is painted repeatedly on the mouse ears. Since DNFB is known for potent stimulant for Th1 [25], the cytokine profile would be Th1 dominant in DNFB-painting mouse. On the other hand, in this study DME induces Th2 dominant condition. Therefore, the regulation of cytokine production by tacrolimus may be changed in the different milieu. Actually, they also have demonstrate that tacrolimus inhibits the production of cytokines by Th2 cells in vitro [24].

Ramierz et al. have reported that pre-exposure of CD4 $^{+}$ lymphocytes to glucocorticoids increase the secretion of Th2-type cytokines, such as IL-4, IL-13, and IL-10, while it suppress the secretion of Th1-type cytokines, such as IFN- γ and TNF- α [26]. This finding also seems to conflict with our result of inhibitory effect of dexamethasone on IL-13 production. However, another report has demonstrated that Th2 cytokine production induced by allergen is inhibited by corticosteroid [27]. Therefore, it can be speculated that steroid induces Th2 cytokine production when the cells are not activated, whereas it inhibits Th2 cytokine production when the cells are activated by certain allergen.

We consider that dust mite allergen is involved in inflammation of AD, but all the pathogenesis

of this disease cannot be explained only by mite allergen. There are many other triggers and aggravating factors in AD. However, our results suggest that PBMCs in AD patients with high mite-specific IgE can react with mite-allergen directly or via antigen presenting cells and can produce Th2-type cytokines such as IL-4, IL-5 and IL-13. Our results would be of help in clarifying the pathogenesis of AD.

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