

Dermatophagoides-induced interleukin-10 production by peripheral blood lymphocytes from patients with asthma in remission

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Interleukin (IL)-10 is a cytokine that regulates inflammatory responses. We studied the role of IL-10 in the development of tolerance to *Dermatophagoides farinae* in asthma patients in remission, since asthma improves in most children during adolescence. The spontaneous production of IL-10 by cultured peripheral blood mononuclear cells (PBMC) was higher in patients with active asthma than in normal subjects. IL-10 production decreased when 1 µg/ml *D. farinae* was added to cultures, but increased again in a dose-dependent manner when higher concentrations of *D. farinae* were added. In patients with remission of asthma, IL-10 production was lower than in patients with active asthma. However, production of IL-10 showed a reciprocal increase in the presence of 1 µg/ml *D. farinae*, and decreased again at 10 and 50 µg/ml *D. farinae*. Such alterations were not observed in normal subjects. Cell lines established from patients asthma in remission showed higher IL-10 production when compared with that by cell lines from normal subjects or patients with active asthma when the cells were stimulated by *D. farinae* at 1 or 10 µg/ml. Neutralization of IL-10 led to revival of the *D. farinae*-specific proliferative response of PBMC from patients in remission, which was otherwise decreased. The increase of IL-10 production stimulated by *D. farinae* was inhibited by addition of an anti-IL-10 antibody. In contrast, antigen-induced interferon (IFN)-γ production, which was increased by *D. farinae* stimulation when patients were in remission, did not increase after treatment with anti-IL-10, although spontaneous IFN-γ production increased to the level seen after *D. farinae* stimulation. The reduced IL-4 production by cells from patients in remission after stimulation with *D. farinae* antigen, which was significantly higher in active patients, was not reversed by neutralization of IL-10. The *D. farinae*-induced IL-10/IL-4 production ratio, but not the IL-10/IL-5 production ratio, may be a significant indicator for evaluation of whether a patient has been in remission. In conclusion, *D. farinae*-specific anergy of T cells is likely to be induced by increased levels of IL-10 and IFN-γ that are initially produced by specific T cells after exposure to relevant mite allergen in patients in remission.

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Abbreviations: IFN-γ, interferon-γ; IL, interleukin; Df, *Dermatophagoides farinae*; OVA, ovalbumin; LPS, lipopolysaccharide; PHA, phytohemagglutinin; PBMCs, peripheral blood mononuclear cells; FEV1, forced expired volume in 1 s; PEF, peak expiratory flow rate; FCS, fetal calf serum; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; ³H-TdR, tritiated thymidine; FITC, fluorescein isothiocyanate.

Interleukin (IL)-10 is a cytokine that regulates the inflammatory response. It is produced by macrophages/monocytes, natural killer cells (1), and both Th1 and Th2 cells (2). IL-10 inhibits the production of interferon (IFN)-γ and IL-2 by Th1 cells, while it suppresses T-cell proliferation and the production of other cytokines by

inhibiting the interaction of CD28 with B7.1 (3). Specific immunotherapy initially induces IL-10-associated T-cell anergy and then modulates the production of immunoglobulin E (IgE), but not IgG4 (4). In contrast, *Dermatophagoides farinae*-induced activation of T cells (as measured by antigen-induced IL-2 responsiveness) or IL-2 synthesis occurs in patients with active asthma, but not in normal subjects, whereas these responses are much weaker when asthma patients are in remission (5). Lymphocytes from patients with active asthma produce less IFN- γ than cells from normal subjects (5). Production of IFN- γ in response to *D. farinae* stimulation is greater in cells from asthma patients in remission than in cells from normal subjects. Therefore, the up-regulation of IFN- γ production after exposure to *D. farinae* might lead to reduced IL-4 secretion (6), which would in turn suppress IgE production and thus improve the clinical status of asthma (5, 7). Accordingly, *D. farinae*-induced IFN- γ production may presumably induce anergy to *D. farinae* in patients with remission of asthma. In this study, we investigated IL-10 production by peripheral blood mononuclear cells (PBMC) from patients with dust mite allergy asthma who had active disease or were in remission.

Materials and methods

Subjects

The subjects were 13 Japanese patients with dust mite allergy-related asthma (Table 1) and four non-allergic volunteers who served as controls. Patients with active disease had recurrent asthma attacks and a positive immediate skin reaction

(defined as an immediate wheal response ≥ 10 mm in mean diameter) in the skin prick tests for house dust mite and *D. farinae* antigen (mite 1; Torii & Co., Ltd, Tokyo, Japan), but not hen's egg antigen (Sigma, St Louis, MO, USA). The serum IgE score, as determined by radioallergo-sorbent testing, for mite antigen (8) ranged from 3 to 5, while that for ovalbumin (OVA; Sigma) was 0.3 ± 0.2 . None of the patients had other allergic diseases, such as atopic dermatitis. The diagnostic criteria for asthma were those of the American Thoracic Society. None of the patients had acute asthma at the time of examination, and none of them were receiving oral or inhaled corticosteroids or antiallergic agents such as ketotifen (9). Patients were enrolled prospectively and were followed until they could be divided into two groups, which were patients in remission (i.e., disease-free for more than 2 yr without medication) and patients with active disease [i.e., more than 10 mild-to-moderate attacks per year despite the use of medications such as theophylline (15–20 mg/kg/day) and β_2 -agonists]. The severity of acute asthma attacks and the overall disease severity were estimated according to the Guidelines for the Diagnosis and Management of Asthma (National Asthma Education Program; Expert Panel Report) released by the National Institutes of Health, USA. A mild attack was defined as normal alertness, absent or mild dyspnea (the patient was able to speak in complete sentences), no or mild intercostal retraction, good skin color, only end-expiratory wheezing, and a peak expiratory flow rate (PEFR) or forced expired volume (FEV) at 70–90% of the predicted value or personal best

Table 1. Characteristics of subjects used in the study

	Controls	Remission	Active
Number of patients	4	7	6
Age (yr)			
Median, ranges	18, 7–34	12, 8–15	10, 7–12
Sex (M:F)	3:1	6:1	3:3
Disease severity	Non-allergy	Disease-free	Mild
Documented		0	more than 10 mild or moderate attacks (per year)*
Medication		—	+ (intermittent or persistent)
Serum IgE (U/ml)	$121 \pm 52^\dagger$	671 ± 132	760 ± 180
<i>Dermatophagoides farinae</i> -IgE CapRAST‡	0 ± 0	4.4 ± 0.9	4.2 ± 0.4
OVA-IgECapRAST‡	0 ± 0	0.3 ± 0.2	0.2 ± 0.2
Eosinophil count (/ μ)	NT	650 ± 154	$1016 \pm 154§$
Skin prick test (<i>Dermatophagoides farinae</i>)	—	+	+
FEV1%	NT	93.2 ± 5.4	96.4 ± 4.9

Controls, non-allergic subjects; remission, patients in remission; active, patients with active disease.

*Per year over the previous 2 yr.

†Mean $\pm$ s.e. NT, not tested.

‡Score.

§Statistically not significant when compared with active.

value. The FEV values of both groups of patients were within normal limits between acute attacks of asthma and during the 4–6 wk before examination, and there was no significant difference between the groups. For at least 2 yr before the study period, asthma was mild-to-moderate in both groups (more than 10 mild or moderate attacks per year, with intermittent or continuous medication). Clinical characteristics of the two patient groups are summarized in Table 1. Informed consent was obtained either from the patient or from the parents in the case of minors.

Cell culture

Peripheral blood mononuclear cells were isolated from heparinized peripheral blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density-gradient centrifugation (10) and were resuspended in RPMI 1640 medium supplemented with 40 µg/ml of gentamycin and 10% heat-inactivated fetal calf serum (FCS; Gibco, Gaithersburg, MD, USA). According to the previously reported method (4, 11), 10^6 PBMC/ml were stimulated in a 24-well plate with serial dilutions of *Dermatophagoides farinae* antigen (Torii & Co., Ltd), 10 µg/ml OVA antigen (Sigma), 20 µg/ml lipopolysaccharide (LPS; Sigma), or 10 µg/ml phytohemagglutinin (PHA; Sigma). For detection of cytokines, the supernatants were harvested on day 3 of LPS or PHA stimulation and on day 5 of *D. farinae* or OVA stimulation to assess maximal production (4, 11). Parallel cultures were done in medium supplemented with a mixture of IL-2 (0.1 units/ml; Takeda Co. Ltd, Tokyo, Japan) and IL-4 (25 ng/ml; Genzyme, Cambridge, MA, USA). After 12 days, the cells were washed three times with phosphate-buffered saline (PBS), and 10^6 cells were restimulated with the same antigen at the same concentration as before in the presence of 10^6 autologous irradiated (3000 rad) PBMC in 24-well tissue culture plates (4, 11). Based on preliminary studies (data not shown), supernatants were harvested after 48 h for determination of maximal IL-10 activity.

To evaluate the proliferative response, 2×10^5 PBMC were stimulated for 6 days with serial dilutions of *D. farinae* in 200 µl of medium. Stimulation was done in 96-well flat-bottomed tissue culture plates (triplicate cultures) and then cultures were pulsed for 24 h with 0.5 µCi of [^{3} H]-thymidine to assess maximal proliferation based on data from preliminary studies (data not shown). Subsequently, the cells were harvested and [^{3} H]-thymidine uptake was quantified by liquid scintillation counting.

The IL-10 was neutralized by adding 10 µg/ml of an anti-IL-10 monoclonal antibody (JES3-19F1 or JES3-9D7; Pharmingen, San Diego, CA, USA) to cell cultures. Rat IgG1 (Sigma Chemical Co.) served as the control antibody.

Measurement of IFN-γ and IL-10

The solid-phase sandwich enzyme-linked immunosorbent assays (ELISAs) for IFN-γ (5, 12) and IL-10 (4, 11) have been described previously. In brief, 96-well microtiter plates (Linbro, Flow Laboratories, Inc., MacLean, VA, USA) were coated with a rabbit antibody for human IFN-γ (PIF-3, Hayashibara Biochemical Laboratories, Inc., Okayama, Japan) and were developed with a mouse monoclonal antibody for human IFN-γ (MIF-3, Hayashibara Biochemical Laboratories, Inc.) and goat biotinylated antibody against mouse IgG (Tago, Inc., Burlingame, CA, USA). Purified human IFN-γ (Japan Chemical Research Pharmaceuticals Co., Ltd, Kobe, Japan) was used as a standard. Results were expressed as units of IFN-γ per milliliter of supernatant with reference to an IFN-γ standard. The sensitivity of this sandwich ELISA was 100 pg/ml.

The IL-10 was measured using the combination of a purified rat antihuman IL-10 monoclonal antibody (JES3-9D7 clone, Rat IgG1; Pharmingen) and a biotinylated rat antihuman IL-10 monoclonal antibody (JES3-12G8 clone, Rat IgG2a; Pharmingen). Results were expressed as nanograms of IL-10 per milliliter of supernatant with reference to a recombinant IL-10 standard (Pharmingen). The sensitivity of the IL-10 ELISA was 50 pg/ml. In both the IFN-γ and IL-10 ELISAs, development was done using peroxidase-labeled streptavidin (Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, MD, USA), while *o*-phenyldiamine HCl (Bethesda Research Laboratories Life Technologies, Inc.) in citrate buffer (pH 5.5) was used as the substrate. Then the absorbance was read at 492 nm with an ELISA reader (Bio-Tek Instruments, Inc., Burlington, VT, USA).

Counting of IL-4-producing cells

Mononuclear cells (1×10^6) were added to serial dilutions of *D. farinae* (Torii & Co, Ltd) and were suspended in 1 ml of RPMI 1640 medium containing 40 µg/ml of gentamycin and 10% heat-inactivated pooled human serum (in order to avoid non-specific appearance of spots) in a culture tube (Falcon Plastic 2054; Becton Dickinson Labware, Lincoln Park, NJ, USA). Then

incubation was done for 3 days (37°C, 10% CO) to achieve maximal production of IL-4, as measured by an ELISPOT assay and reported elsewhere (5). Briefly, 96-well microplates (Linbro, Flow Laboratories, Inc.) were coated overnight at 4°C with a purified monoclonal antibody for IL-4 (380-1, Ono Chemical Industries, Osaka, Japan) at a concentration of 30 µg/ml (100 µl/well). Control wells were coated with PBS containing 5% FCS. All wells were then blocked with 200 µl of 0.25% gelatin (Wako Pure Chemical Industries, Ltd, Tokyo, Japan) in PBS for 1 h at 37°C. Cells (1×10^5 /well) were resuspended in RPMI 1640 medium containing 3% FCS and were added to each well (100 µl/well), after which incubation was done for 16 h (37°C, 10% CO). Then the cells were washed three times with PBS and incubated in 1% BSA-PBS for 2 h at room temperature with 100 µl of a rabbit polyclonal antibody for human IL-4 (4 µg/ml, BL-4, Genzyme Corp., Boston, MA, USA). Next, the plates were washed three times with PBS and incubated for 2 h at room temperature in 1% BSA-PBS (100 µl/well) with alkaline phosphatase-conjugated goat antirabbit IgG (diluted 1:1000, 65-6122, Zymed Laboratories, Inc., San Francisco, CA, USA). Subsequently, the plates were washed three times with PBS and refilled with 100 µl of 1 mg/ml alkaline phosphatase substrate solution, which contained 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma) dissolved at 40°C in 0.6% (w/v) agarose (Electlan, 44250, BDH, Poole, England, UK), as well as 0.1 M 2-amino-2-methyl-1-propanol, 0.02% (v/v) Triton X-405 (BDH) and 5.0 mM MgCl. Spots representing single IL-4-secreting cells that developed during 4 h of incubation at 37°C were counted under an inverted microscope. The specificity of the ELISPOT assay for detecting IL-4-secreting cells was determined by an inhibition test using an anti-IL-4 antibody.

IL-5 production and measurement of its activity

The PBMC (1×10^6 /ml) were cultured with serial dilutions of *D. farinae* (Torii & Co.) in RPMI 1640 medium containing 40 µg/ml gentamycin and 5% FCS (Sigma). After incubation for 3 days, supernatants were collected and maximal IL-5 activity was determined with a bioassay (5, 13) using FDC-h5R cells (13) that were kindly provided by Dr Kiyoshi Takatsu (Institute of Medical Science, Tokyo University). Briefly, FDC-5hR cells were treated with 100 U/ml of recombinant mouse IL-3 (Genzyme Corp.) and then cultured for 2 days in RPMI 1640 medium supplemented with 5% FCS in a 96-well microplate (Nunc, Roskilde, Denmark) (37°C, 10% CO). Next, 1×10^4

FDC-5hR cells were added to the supernatants and cultured for 3 days. Then, 0.5 µCi of ^3H -TdR was added to each well, after which culture was continued for an additional 24 h before harvesting of cells and measurement of ^3H -TdR uptake with a liquid scintillation counter. Recombinant IL-5 (Genzyme) was used as the standard and results were expressed with reference to it. The sensitivity of this assay was 2 pg/ml.

To assess the IL-5-dependent proliferation of FDC-h5R cells, 1×10^4 cells were added to recombinant IL-5 or to culture supernatants obtained from *D. farinae*-stimulated PBMC of asthma patients, and then were cultured for 3 days with or without rat antihuman IL-5 antibody (Pharmingen). The ^3H -TdR uptake was quantified after incubation for an additional 24 h.

Statistical analysis

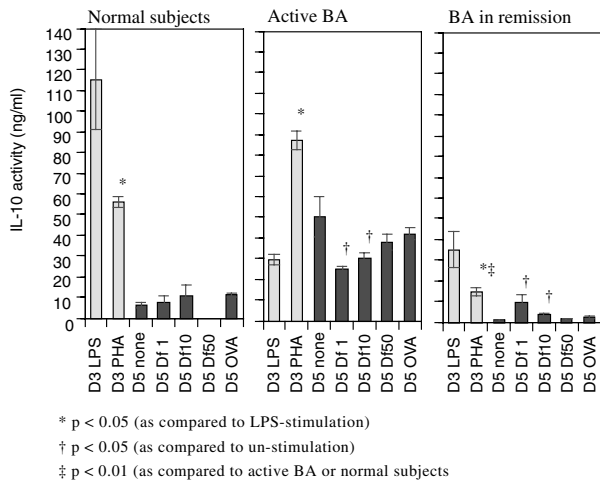
Statistical comparisons with control data were carried out using analyzed with the Mann-Whitney non-parametric test. Results are given as the mean $\pm$ s.e. A p-value < 0.05 was considered to indicate statistical significance.

Results

IL-10 production

Upon stimulation with LPS or PHA, IL-10 was produced by PBMC cultured for 3 days (Fig. 1). LPS-stimulated more IL-10 production by cells from normal subjects than by cells from patients with active asthma or patients who had asthma in remission. IL-10 production by cells from active asthma patients was higher after stimulation with PHA than LPS, while production was low when cells from patients with remission of asthma were tested.

Unstimulated PBMC from normal subjects showed little IL-10 production after culture for 5 days. The paucity of IL-10 production by these cells not increased by treatment with *D. farinae*. PBMC from active asthma patients showed higher production of IL-10 than PBMC from normal subjects. IL-10 production was decreased when 1 µg/ml of *D. farinae* was added to cultures, whereas it was increased concentration dependently when the cells were cultured with 10 or 50 µg/ml of *D. farinae* (Fig. 1). Although IL-10 production was increased following culture with higher concentrations of *D. farinae*, it does not reach the level produced by unstimulated cells. After stimulation with OVA, IL-10 production was comparable with that by unstimulated cells. Production of IL-10 by unstimulated



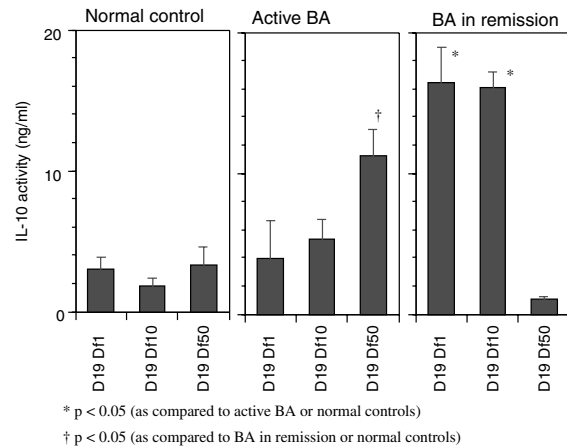
A representative result of four independent experiments is shown because the individual data varied, although showing the same trend.

Fig. 1. Interleukin (IL)-10 production by peripheral blood mononuclear cells (PBMC). PBMC (1×10^6 /ml) were stimulated with serial dilutions of *Dermatophagoides farinae* antigen, ovalbumin (OVA), lipopolysaccharide (LPS), or phytohemagglutinin (PHA). Supernatants were harvested on day 3 of LPS- or PHA-stimulated culture and on day 5 of *D. farinae*- or OVA-stimulated culture. IL-10 activity was determined by enzyme-linked immunosorbent assay (ELISA).

cells from the asthma patients in remission was lower than by unstimulated cells from active asthma patients or normal subjects (Fig. 1). IL-10 production was increased when $1 \mu\text{g}/\text{ml}$ of *D. farinae* was added and culture was done for 5 days. When 10 or $50 \mu\text{g}/\text{ml}$ of *D. farinae* was added, however, IL-10 production was actually lower (Fig. 1). In cell lines established from patients with asthma in remission, production of IL-10 was similarly increased by stimulation with 1 or $10 \mu\text{g}/\text{ml}$ of *D. farinae* when compared with that by cells from active asthma patients or normal subjects, whereas IL-10 production was low after incubation with $50 \mu\text{g}/\text{ml}$ of *D. farinae* (Fig. 2). In contrast, IL-10 production by cell lines from normal subjects and active asthma patients was very low after stimulation with 1 or $10 \mu\text{g}/\text{ml}$ of *D. farinae* (Fig. 2), while the production of IL-10 by cells from active asthma patients was higher than by cells from patients in remission or normal subjects after stimulation with $50 \mu\text{g}/\text{ml}$ of *D. farinae* (Fig. 2). In cultures of unstimulated cell lines, IL-10 production was minimal (data not shown).

Comparison of cytokine production

Production of IL-4 (measured by counting the number of immunoreactive cells) and IL-5 was



A representative result of four independent experiments is shown because the individual data varied, although showing the same trend.

Fig. 2. Interleukin (IL)-10 production by *Dermatophagoides farinae*-stimulated T cell lines. Peripheral blood mononuclear cells (PBMC) (1×10^6 /ml) were stimulated with serial dilutions of *D. farinae* and then cultured for 5 days. Then the cultures were expanded in medium supplemented with a mixture of IL-2 and IL-4. After 12 days, 10^6 cells were restimulated with the same antigen at the same concentrations as before in the presence of 10^6 autologous irradiated (3000 rad) PBMC. Supernatants were harvested after 48 h for determination of IL-10 activity by enzyme-linked immunosorbent assay (ELISA).

observed after stimulation of PBMC from active asthma patients with 1 or $10 \mu\text{g}/\text{ml}$ of *D. farinae*, but not after stimulating cells from patients with remission of asthma or cells from normal subjects, as reported previously (Fig. 3) (5, 14). To investigate the association of IL-10 production with the production of Th2 cytokines (including IL-5 and IL-4), the stimulation index of each cytokine at the time of maximal production was compared with the mean untreated value. The stimulation index for IL-10 was markedly increased by both 1 and $10 \mu\text{g}/\text{ml}$ of *D. farinae* in the case of cells from asthma patients in remission (Fig. 4a). However, there was no significant difference between cell from normal subjects and active asthma patients. The IL-10/IL-5 production ratio was similarly increased by 1 and $10 \mu\text{g}/\text{ml}$ of *D. farinae* in the patients with remission of asthma (Fig. 4b), while no significant difference was observed between cells from normal subjects and active patients. In contrast, the IL-10/IL-4 production ratio was markedly increased by $1 \mu\text{g}/\text{ml}$ of *D. farinae* in cells from patients with remission of asthma, and it was also higher in cells from normal subjects than in cells from active patients (Fig. 4c). After stimulation with $10 \mu\text{g}/\text{ml}$ of *D. farinae*, the IL-10/IL-4 ratio was higher in cell from remission patients than in cells from active patients as well, while no

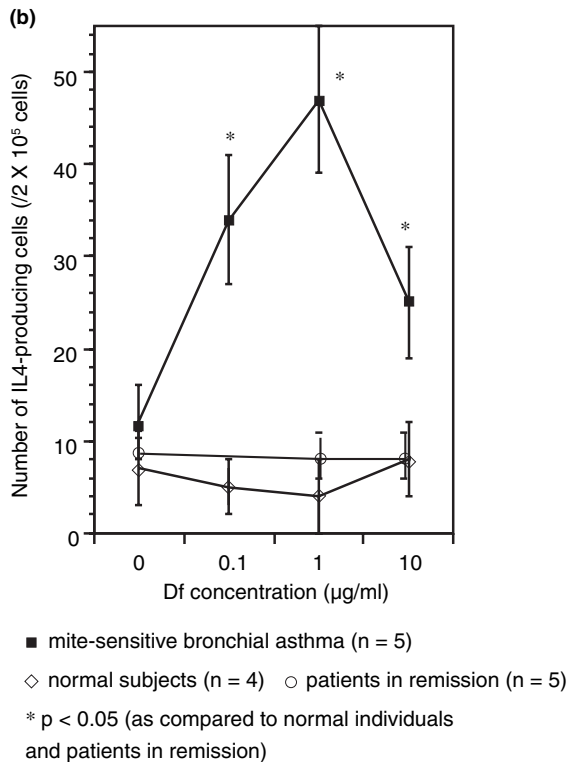
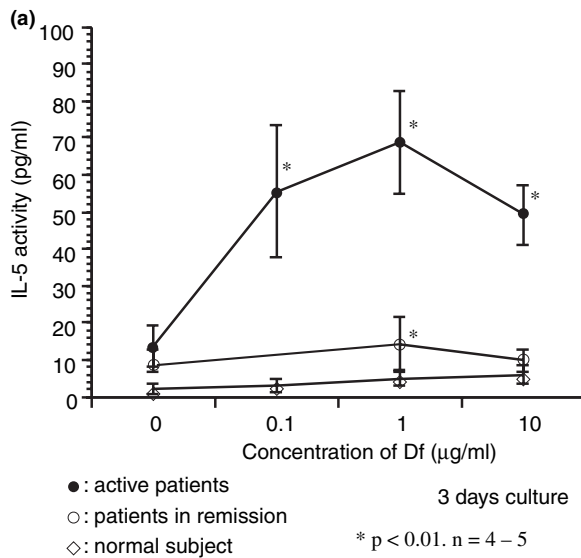


Fig. 3. (a) Interleukin (IL)-5 production by peripheral blood mononuclear cells (PBMC) stimulated with *Dermatophagoides farinae*. PBMC (1×10^6 /ml) were cultured with serial dilutions of *D. farinae*. After incubation for 3 days, supernatants were collected, and IL-5 activity was determined with a bioassay using FDC-h5R cells. FDC-5hR cells (1×10^4 cells) were added to each supernatant and then cultured for 3 days. Then $0.5 \mu\text{Ci}$ of [^3H]-thymidine was added to each well, after which cultures were incubated for an additional 24 h before harvesting. The [^3H]-thymidine uptake was measured with a liquid scintillation counter. (b) IL-4 production by peripheral blood mononuclear cells stimulated with *D. farinae*. PBMC (1×10^6 /ml) were treated with serial dilutions of *D. farinae* and cultured for 3 days. Then an ELISPOT assay was done to count the cells producing IL-4.

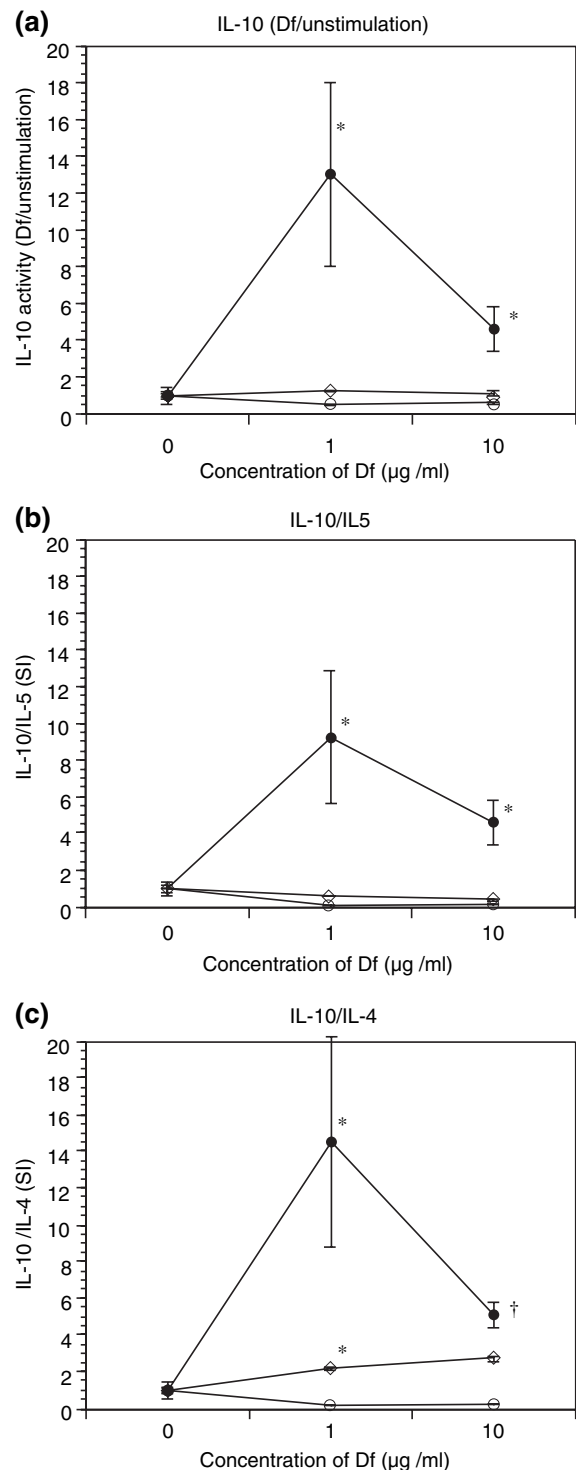


Fig. 4. Ratio of interleukin (IL)-10 production to IL-5 or IL-4 production. In each group of subjects, to investigate the correlation of IL-10 production with the production of Th2 cytokines (IL-5 and IL-4), the stimulation index of each value vs. the mean untreated value was compared.

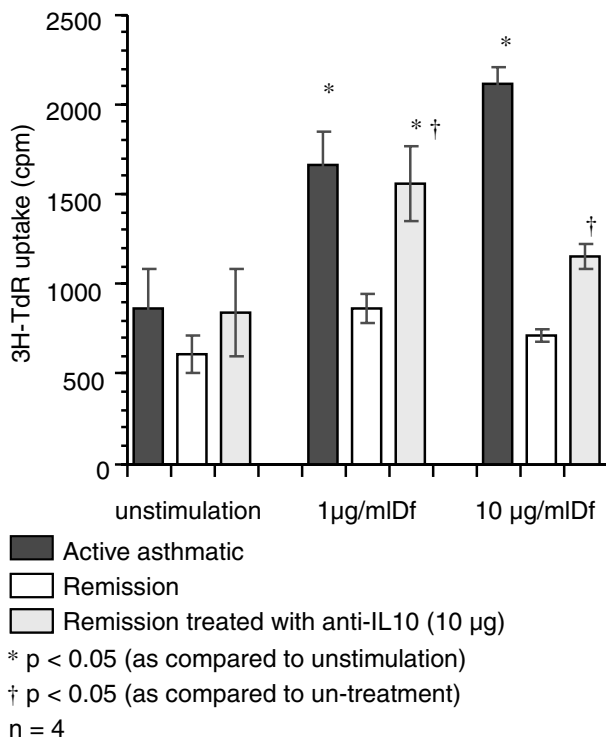


Fig. 5. Neutralization of interleukin (IL)-10 restores the *Dermatophagoides farinae*-induced proliferation of cells from asthma patients in remission. To evaluate proliferative responses, 2×10^5 peripheral blood mononuclear cells (PBMC) were stimulated with serial dilutions of *D. farinae* in 200 µl of medium in 96-well flat-bottomed tissue culture plates for 6 days and then pulsed for 24 h with 0.5 µCi of [³H]-thymidine. Then the cells were harvested and [³H]-thymidine uptake was quantified by liquid scintillation counting. A representative result of four independent experiments is shown (all data showed the same trend).

significant differences were noticed between cells from normal subjects and active patients (Fig. 4). These results suggested that the IL-10/IL-4 production ratio was a possible indicator for evaluating the likelihood of patients experiencing asthma remission, provided that the test cells were stimulated with the optimal concentration of *D. farinae*.

Role of IL-10 in lymphocyte proliferation and cytokine production in patients with remission of asthma

When the effect of IL-10 on lymphocyte proliferation was evaluated after stimulation with *D. farinae*, [³H]-thymidine uptake was lower in cells from patients with remission than in cells from active asthma patients (Fig. 5) or normal subjects (data not shown). Simultaneous addition of anti-IL-10 to the *D. farinae*-stimulated cultures led to increased proliferation of cells from the patients with remission of asthma (Fig. 5).

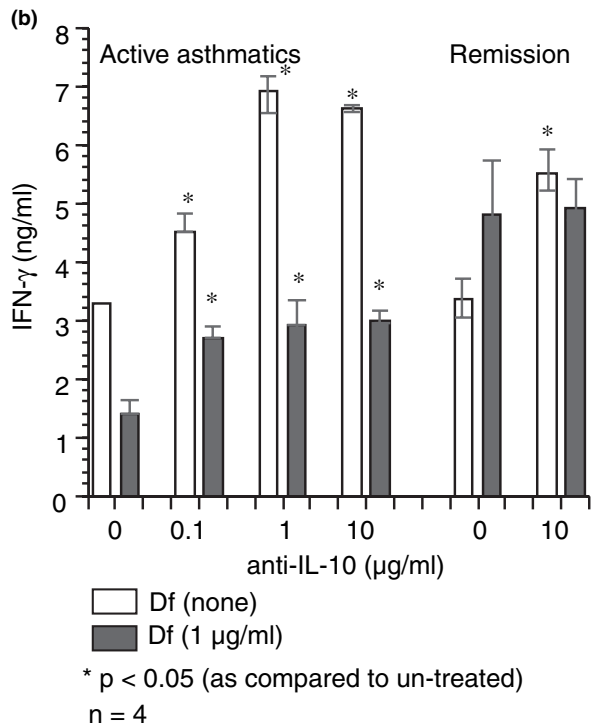
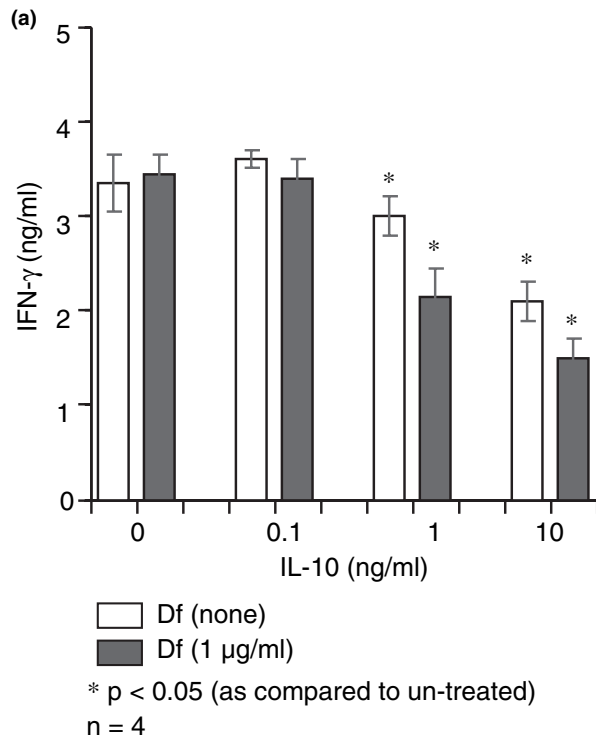


Fig. 6. (a) Interleukin (IL)-10 inhibits interferon (IFN)-γ production. Normal peripheral blood mononuclear cells (PBMC) were treated with recombinant IL-10 with or without *Dermatophagoides farinae* stimulation, and then cultured for 5 days. The IFN-γ activity of the supernatants was measured by enzyme-linked immunosorbent assay (ELISA). (b) Neutralization of IL-10 restores IFN-γ production. A rat anti-IL-10 monoclonal antibody (mAb) (JES3-19F1 or JES3-9D7, 10 µg/ml) was added to *D. farinae*-stimulated cells and incubation was done for 5 days. Then the IFN-γ activity of the supernatants was measured by ELISA.

The increase of IL-10 production stimulated by 1 or 10 $\mu\text{g/ml}$ of *D. farinae* in cells from patients in remission was markedly inhibited by treatment with the anti-IL-10 antibody (data not shown).

The IFN- γ production by both unstimulated and *D. farinae* (1 $\mu\text{g/ml}$)-stimulated PBMC from normal subjects was inhibited by the addition of recombinant IL-10 (Fig. 6a). IFN- γ production by *D. farinae* (1 $\mu\text{g/ml}$)-stimulated PBMC from active asthma patients was decreased compared with that by unstimulated cells, as shown previously (5), and was increased by treatment with an anti-IL-10 antibody (Fig. 6b). Spontaneous production of IFN- γ was also markedly increased by exposure to the antibody. An increase of IFN- γ production was observed when PBMC from patients in remission were exposed to 1 $\mu\text{g/ml}$ of *D. farinae* (5) and was not changed by treatment of cells with the anti-IL-10 antibody, whereas this antibody caused IFN- γ production by unstimulated PBMC to increase to the same level as that by *D. farinae*-stimulated cells (Fig. 6b).

A decrease of IL-4 production by PBMC stimulated with 1 or 10 $\mu\text{g/ml}$ of *D. farinae* was observed in cells from the patients in remission as well as in cells from normal subjects, and was not changed by treatment with the anti-IL-10 antibody (data not shown).

Discussion

We previously reported that *Dermatophagoides*-specific T-cell anergy was associated with increased IFN- γ production (5). Akdis et al. also reported that increased IL-10 production could lead to an anergic state during bee venom-specific immunotherapy (4, 11). Accordingly, we studied the role of IL-10 in the development of tolerance to *D. farinae* in mite-sensitive asthma patients, as asthma improves markedly in most children during adolescence and only a small minority still show significant symptoms by the age of 20 yr (5, 15–17).

After stimulation with an optimal concentration (1 $\mu\text{g/ml}$) of *D. farinae*, lymphocytes from our asthma patients with remission showed anergy along with up-regulated IL-10 and IFN- γ production. This may be a similar state to the IL-10-associated T-cell anergy that occurs during bee venom-specific immunotherapy (4, 11).

When cell lines established from patients with remission of asthma were stimulated with 1 and 10 $\mu\text{g/ml}$ of *D. farinae*, IL-10 production was increased when compared with that by cell lines established from normal subjects and active asthma patients. Similarly, IL-10 production by

cultured PBMC from patients in remission was increased after stimulation with an optimal concentration (1 $\mu\text{g/ml}$) of *D. farinae*. In contrast, the *D. farinae*-specific proliferative response of PBMC was decreased when compared with that of PBMC from active asthma patients.

Neutralization of IL-10 by addition of an anti-IL-10 antibody resulted in the restoration of these *D. farinae*-specific proliferative responses to cells from the asthma patients in remission and IL-10 production was also inhibited.

The IFN- γ production by PBMC from normal subjects was inhibited after addition of IL-10 (Fig. 6a). In active asthma patients, IFN- γ production was decreased after stimulation with *D. farinae*, but was augmented by treatment with anti-IL-10 antibody, as shown in Fig. 6b. However, IFN- γ production by PBMC from patients in remission, which was increased by *D. farinae* stimulation (5), was not changed by treatment with the anti-IL-10 antibody (probably due to the lower IL-10 level in the culture medium when compared with that for active asthma patients), although spontaneous IFN- γ production was increased by the antibody to the level seen after *D. farinae* stimulation. These results indicate that even a low concentration of IL-10, such as that produced endogenously by unstimulated cells from patients in remission, can down-regulate spontaneous production of IFN- γ , whereas the relatively high levels of IL-10 produced by *D. farinae* stimulation could not affect IFN- γ production from cells activated by *D. farinae*. Thus, the relatively small increase of IL-10 after *D. farinae* stimulation presumably fails to inhibit the augmentation of IFN- γ production. In the case of bee venom-specific T-cell anergy, antigen-stimulated IFN- γ production was also not changed significantly by neutralization of IL-10 (4). These data suggest that *D. farinae*-specific anergy arises through the co-existence of increased IL-10 and IFN- γ production in patients with remission of asthma. *Dermatophagoides farinae*-induced IFN- γ production by cells from these patients showed an increase independent of IL-10, although IL-10 was able to regulate IFN- γ production by cells from normal subjects. Thus, the *D. farinae*-specific anergic state is likely to result in modulation of the production of Th2 cytokines, such as IL-4 (5).

Anergic T cells from the peripheral blood are reported to be reactivated and to produce Th1 cytokines after culture with IL-2 or IL-15 (11). This report supports the increased production of IFN- γ by *D. farinae*-stimulated PBMC from our asthma patients in remission.

The IL-4 production did not recover after neutralization of IL-10 under the same conditions, indicating that IL-10 did not directly down-regulate IL-4 production. This finding is supported by a report that IL-10 has a direct effect on B cells, in which it inhibits total IgE synthesis and simultaneously enhances IgG4 synthesis, although these processes are usually mediated by IL-4 (4, 18). Thus, the decrease of IL-4 production by cells from our patients in remission was probably influenced by IFN- γ , which was increased by *D. farinae* stimulation, but not by IL-10.

This study suggested that anergy was likely to be induced in T cells after activation by *D. farinae* in the absence of accessory signals (19), a state that is usually characterized by suppressed proliferative and cytokine responses. The inhibitory effect of IL-10 on T cells is only observed in antigen-presenting cell-dependent systems, suggesting that IL-10 may block the CD28/B7.1 interaction and subsequent costimulatory signaling pathways (3, 19). However, we found that IFN- γ production by *D. farinae*-stimulated cells, which resulted in the down-regulation of IL-4 production, was not regulated by IL-10. Actually, IL-10 production by the cells from patients in remission was lower than that by cells from active asthma patients or normal subjects, even after stimulation with an optimal concentration of *D. farinae*. These lower levels of IL-10 could not suppress IFN- γ , although IL-10 was involved in the decreased proliferative response of lymphocytes to *D. farinae*. Thus, the number of *D. farinae*-reactive T cells secreting IL-10 might decrease when patients are in remission. However, *D. farinae*-stimulated T cell lines (clones) from the patients in remission produced more IL-10 than clones from active asthma patients. This indicates that *D. farinae*-activated T cells secreting IL-10 were maintained in during remission by recurrent exposure to the antigen, which is likely to be involved in the patient experiencing asthma remission.

In active asthma patients, IL-10 production was higher after PHA stimulation than that seen patients with remission, whereas addition of *D. farinae* at a dose which was optimal for production of IL-4 and IL-5 caused the down-regulation of IL-10 production. These results suggest that modulation of IL-10 production by the relevant antigen is likely to be involved in determining disease severity, although the ability of the T cells to secrete IL-10 after stimulation by agents such as PHA was conserved.

In contrast, it was previously reported that IL-10 production is initially triggered in

antigen-specific T cells, followed by antigen-presenting cells. IL-10 plays an important role in both the induction and maintenance of specific T-cell tolerance (20, 21), and there is a subset of regulatory T cells that secrete high levels of IL-10 and are involved in blocking antigen-specific T-cell responses (22). *Dermatophagoides farinae*-induced T cell lines that produce IL-10 and induce anergy, as demonstrated in our patients with remission of asthma, may be similar to these regulatory cells. These results suggest that IL-10 produced by cells such as antigen-presenting cells stimulated with the relevant antigen or LPS is unlikely to be the only cytokine involved in inhibiting antigen-specific responses. Production of IL-10 by cells stimulated with LPS was higher in normal subjects than in the asthma patients, but was similar between patients with active asthma and patients in remission. This suggests that high IL-10 secretion by antigen-presenting B cells and monocytes may potentiate and maintain the anergy of specific T cells in normal persons, as occurs in naturally anergic individuals who are chronically exposed to mite antigen. However, the change of T cells to IL-10-producing cells may be of great importance for remission of asthma, because *D. farinae*-specific T cell lines from the patients in remission showed higher IL-10 production when compared with the patients with active asthma.

Dermatophagoides farinae-stimulated lymphocyte proliferation was increased by high levels of IL-10 in active asthma patients, while neutralization of IL-10 allowed the restoration of *D. farinae*-specific proliferative responses in the patients with remission. The influence of IL-10 on lymphocyte proliferation was probably blocked by simultaneous production of growth-promoting cytokines such as IL-4 and IL-2, because IL-4 production is not regulated by IL-10.

Finally, we evaluated parameters for assessing both the clinical and immunologic remission of asthma. Since neutralization of IL-10 did not influence IL-4 production in this study and IL-10 has been confirmed to down-regulate IL-5 production (3), the IL-10/IL-4 and IL-10/IL-5 production ratios were investigated. The IL-10/IL-4 ratio, but not the IL-10/IL-5 ratio, seemed to be a useful indicator for evaluating whether or not a patient had been in remission, as the differences among the three groups (active asthma patients, patients in remission, and normal subjects) were significant if the cells were stimulated with an optimal concentration of *D. farinae*.

In patients with remission of asthma during adolescence, cells exposed to a relevant antigen (*D. farinae*) responded with increased IL-10 and

IFN- γ production. These findings suggest that induction of T-cell anergy can govern inflammatory cytokine production associated with the normal immune response to an allergen present in the local microenvironment. (23, 24). Although longitudinal studies of the same patients throughout remission would have been more informative, the alterations of cytokine production shown by the present study appear to be involved in the immunologic mechanisms leading to the remission of asthma related to mite antigen allergy.

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