

IL-12 affects *Dermatophagoides farinae*-induced IL-4 production by T cells from pediatric patients with mite-sensitive asthma

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Background: IL-12 is a critical cytokine in the regulation of immune responses produced by phagocytic cells exposed to microorganism infection.

Objective: We sought to study the effect of low doses and high doses of IL-12 on T_{H1} versus T_{H2} cytokine expression to elucidate the etiology of mite antigen-sensitive bronchial asthma in infants.

Methods: We studied the effect of IL-12 on *Dermatophagoides farinae* (Df) antigen-induced IL-4 production and subsequent production of IgE by PBMCs from pediatric patients with asthma.

Results: Simultaneous addition of 1 to 10 ng/mL IL-12 to cultures enhanced Df-induced IL-4 production, although low doses (0.05 to 0.1 ng/mL) of IL-12 downregulated IL-4 production. Endogenous IL-12 is required for such production. These phenomena were not observed in Df-stimulated control PBMCs. In contrast, on stimulation with the same dose of Df, IFN- γ production by patient PBMCs was enhanced in a dose-dependent fashion by addition of IL-12. Quantification analysis of RT-PCR-amplified DNA fragments by laser-induced fluorescence showed that a high dose of IL-12 augments mRNA expression for IL-4 protein synthesis, whereas a low dose of IL-12 inhibits IL-4 mRNA expression, and that the signal of mRNA for IFN- γ protein synthesis was increased on Df stimulation in a dose-dependent fashion. Df-induced in vitro production of IgE and Df-specific IgE in serum from severe combined immunodeficient mice reconstituted with PBMCs were increased by treatment with high doses of IL-12, whereas low doses of IL-12 inhibited that production. The combined results indicate that at a low dose of IL-12, IL-4 and IFN- γ production was regulated reciprocally; however, at high doses of IL-12, cells produced IL-4 and IFN- γ simultaneously, and neither cytokine was regulated.

Conclusion: Low-dose and high-dose IL-12 induce T_{H1} responses, and high-dose IL-12 induces both T_{H1} responses and T_{H2} or T_{H0} responses. Consequently, the IL-4 production may overcome T_{H1} -type cell activation of IgE production in patients

with mite-sensitive bronchial asthma. (J Allergy Clin Immunol 1999;103:850-8.)

Key words: IL-12, *Dermatophagoides farinae*-specific IgE, IL-4 mRNA, pediatric bronchial asthma

Allergic disease is caused by dysregulation of the synthesis of such cytokines as IL-4, IL-5, and IL-10 (but not IFN- γ) by allergen-activated CD4⁺ T cells.¹⁻⁶ IL-12, a critical cytokine in the regulation of immune responses produced by phagocytic cells, natural killer (NK) cells, mast cells/basophils, and $T\gamma\delta$ cells,⁷⁻⁹ enhances T-cell proliferation,^{10,11} induces cytotoxic T lymphocyte and NK lytic activity¹² and costimulatory signals,¹³ and augments IFN- γ production by T and NK cells.^{14,15} It is rapidly secreted by phagocytic cells exposed to bacterial or viral infection.¹⁴ In naive T cells, IL-12 initiates the differentiation of CD4⁺ T cells into T_{H1} cells secreting IFN- γ .¹⁶

In allergen-specific human CD4⁺ T lymphocytes from allergic donors stimulated in vitro, IL-12 inhibited the development of IL-4 and IL-10 synthesis on stimulation by allergen, whereas it enhances synthesis of IFN- γ and IL-2.¹⁷ However, IL-12 exerts differential effects on the maturation of "naive" human CD4⁺ T cells isolated from umbilical cord blood or from the blood of healthy adults. In neonates after priming in the presence of IL-12, cells develop into effector cells, which produce high levels of IL-4 in addition to IFN- γ , whereas unstimulated neonatal T cells preincubated with IL-12 behave like adult T cells, which acquire a T_{H1} phenotype after stimulation in the presence of IL-12.¹⁸ In established IL4-producing T_{H0} - and T_{H2} -like human T-cell clones, IL-12 is known to potentiate the production of IL-4.¹⁹

Asthmatic children have been shown to have the disease early in life, with most cases being largely or totally induced by mite antigen.²⁰⁻²² Initiation of the disease may sometimes be involved in infection by viruses such as respiratory syncytial virus (RSV).⁶ IL-12 secreted by the infected cells promotes the maturation of infant, but not adult, T cells into IL-4 producers and may explain initiation of mite antigen-induced bronchial asthma (BA) in early infancy, as well as the increased susceptibility to intracellular pathogens.¹⁸ In this article, to elucidate the pathogenesis of mite antigen-induced BA in infants, the effect of IL-12 on *Dermatophagoides fari-*

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Abbreviations used

BA:	Bronchial asthma
Df:	<i>Dermatophagoides farinae</i>
ELISPOT:	Enzyme-linked immunospot assay
NK:	Natural killer
RSV:	Respiratory syncytial virus
SCID mice:	Severe combined immunodeficient mice

nae (Df) antigen-induced IL-4 production and subsequent production of IgE by PBMCs from the patients was studied.

METHODS

Subjects

PBMCs were obtained from 6 Japanese patients with BA (age: median, 6 years; range, 3 to 10 years; sex [M:F], 3:3). Five age-matched (age: median, 7 years; range, 3 to 13 years; sex [M:F], 3:2) and 3 adult healthy subjects were used as the control group. Patients with active disease had recurrent episodes of asthmatic attacks and positive immediate skin reactions (as defined by an immediate wheal response to a skin prick test; positive response, ≥ 10 mm mean diameter) to house dust mite and Df antigen (mite 1; Torii & Co, Ltd, Tokyo, Japan). Serum levels of total IgE were 1139 ± 347 IU/mL (mean $\pm$ SEM; control subjects, 124 ± 39 IU/mL), and serum IgE scores against mite antigen,²³ as determined by RAST, varied from 3 to 5 (control subjects, 0). The diagnostic criteria for BA used were those defined by the American Thoracic Society. No patient had an asthmatic attack at the time of the examination, and no patient was taking oral corticosteroids or any anti-allergy agent, such as ketotifen.²⁴ The patients had more than 10 mild or moderate attacks per year despite use of medications such as theophylline (15 to 20 mg/kg/day), β_2 -agonists, or some combination thereof. The severity of acute exacerbations of asthma and disease severity were estimated according to the Guidelines for the Diagnosis and Management of Asthma in the National Asthma Education Program Expert Panel Report by the National Institutes of Health. A mild attack was defined as normal alertness, absent or mild dyspnea (patient was able to speak in complete sentences), absent or mild intercostal retraction, good skin color, end-expiratory wheezing only, and 70% to 90% of predicted or personal best peak expiratory flow rate or FEV₁. Informed consent was obtained from each patient or from a parent. These investigations were formally approved by our institutional review board.

Mice

C.B-17-SCID mice were purchased from CLEA Japan Corp (Tokyo, Japan) and maintained under specific pathogen-free conditions without prophylactic antibiotic treatment in the animal center of Saitama Medical School. Eight- to 12-week-old mice were used in this study.

IL-4 production and counting of cells secreting IL-4

Mononuclear cells (1×10^6) were added to serial dilutions of Df antigen (Torii & Co, Ltd) or ovalbumin (Sigma, St Louis, Mo), suspended in 1 mL of RPMI-1640 medium containing 40 μ g/mL gentamycin and 10% heat-inactivated pooled human serum in a culture tube (Falcon Plastic 2054), and cultured for 2 days at 37°C in a 10% CO₂ atmosphere. An enzyme-linked immunospot (ELISPOT) assay was done, as reported elsewhere,^{6,25} to evaluate the ability of these cells to produce IL-4. The specificity of the ELISPOT assay for detecting IL-4-secreting cells was determined by inhibition with

soluble antibody against IL-4. Specific antigen-induced IL-4 activity in the culture supernatants was under minimum detection of sandwich ELISA systems, as mentioned in the next section, probably because of extremely small amounts of IL-4 secreted. Therefore IL-4 activity was evaluated by using IL-4-secreting cells.

To evaluate the effect of IL-12 on Df-induced IL-4 production, the mononuclear cells from patients with mite-sensitive BA were added to serially diluted recombinant IL-12 (R&D Systems, Inc, Minneapolis, Minn) simultaneously with stimulation by a 1 μ g/mL dose of Df antigen (an optimal dose for IL-4 production) and then cultured for 48 hours for IL-4 production. To further evaluate the requirement of endogenous IL-12 for Df-induced IL-4 production, Df-stimulated cells were added to serially diluted anti-IL-12 antibody (R&D Systems, Inc) and then cultured for 48 hours to induce IL-4-secreting cells. An ELISPOT assay was done, as described above, to evaluate the ability of these cells to produce IL-4.

IFN- γ production and assay

To examine the effect of IL-12 on Df-induced IFN- γ production, mononuclear cells (1×10^6) from patients with mite-sensitive BA were added to serially diluted recombinant IL-12 on stimulation with 0.1 or 1 μ g/mL doses of Df antigen in 1 mL of RPMI-1640 medium containing 40 μ g/mL gentamycin and 2% heat-inactivated pooled human serum in a culture tube (Falcon Plastic 2054). Then the cells were cultured for 3 days at 37°C in a 10% CO₂ atmosphere. Culture supernatants from the cells were harvested and assayed for IFN- γ by using a slightly modified version of a solid-phase ELISA, as reported elsewhere.^{6,25} Purified human IFN- γ (Japan Chemical Research Pharmaceuticals Co, Ltd, Kobe, Japan) was used as the standard. Results are expressed as units of IFN- γ per milliliter of supernatant with reference to a standard IFN- γ . The sensitivity of this sandwich ELISA assay was 1 U/mL.

Total IgE production by PBMCs

Patient PBMCs (1×10^6) were added to 1 μ g/mL Df antigen suspended in 1 mL of RPMI-1640 medium containing 40 μ g/mL gentamycin and 10% heat-inactivated pooled human serum in a culture tube (Falcon Plastic 2054) and cultured for 9 days at 37°C in a 10% CO₂ atmosphere. To examine the effect of IL-12 on Df-induced IgE production, patient cells were added to serially diluted recombinant IL-12 simultaneously with stimulation by 1 μ g/mL Df antigen and then cultured for 9 days. Supernatants were collected for IgE activity and stored at -20°C until use.

Df-specific IgE production in SCID mice reconstituted with human PBMCs

Patient PBMCs (1×10^6) were suspended in the presence of 10 μ g/mL Df antigen in 1 mL of serum-free RPMI-1640 medium in a culture tube (Falcon Plastic 2054). After 4 hours' culture at 37°C in a 10% CO₂ atmosphere, the cells were washed 4 times with PBS and injected intraperitoneally in 500 μ L of PBS into SCID mice by means of a 23-gauge needle. In each experiment 1×10^7 cells were injected. To examine the effect of IL-12 on Df-induced specific IgE production, the Df-stimulated cells were treated with 0.05 or 10 ng/mL recombinant IL-12 and injected intraperitoneally into SCID mice. The mice sera were collected for Df-specific IgE activity on the day indicated in the Results section.

Measurement of human total and Df-specific IgE

Total IgE activity for the culture supernatants and Df-specific IgE activity for the serum were measured by using a slightly modified version of a solid-phase ELISA reported elsewhere.²⁶ Microtiter plates (96-well; Linbro, Flow Laboratories, Inc,

MacLean, Va) were coated with 100 μ L/well goat anti-human IgE antibody (Cappel, Malvern, Pa) to measure total IgE or with 50 μ g/mL Df to measure Df-specific IgE in NaHCO₃ buffer (0.1 mol/L, pH 9.6) overnight at 4°C in a humidified box. A total of 100 μ L of the supernatant or 10-fold diluted serum from SCID mice per well was assayed. Purified human IgE derived from human myeloma cells or human serum with a high titer of Df-specific IgE was used as the standard. The goat anti-mouse IgG antibody used as the second antibody was passed through a goat Ig- and human Ig-coupled Sepharose 4B column to prevent nonspecific binding with goat and human Ig, after which it was diluted 200-fold for use. Results are expressed as picograms per milliliter of IgE with reference to a standard Ig. The sensitivity of this sandwich ELISA was approximately 40 pg/mL for total and specific IgE. Levels of specific IgE against Df antigen in human serum were determined by RAST.²³

Reverse transcription

Patient PBMCs treated with 0.05 or 10 ng/mL IL-12 were cultured for 48 hours simultaneously with stimulation by 1 μ g/mL Df antigen, and then total RNA was extracted with Trizol reagent (Gibco BRL, Gaithersburg, Md), according to the manufacturer's instructions, in a variation of a single-step acid guanidinium-thiocyanate-phenol-chloroform RNA extraction method.²⁷

The total RNA (0.5 μ g) and standard RNA at various concentrations were reverse transcribed in a total volume of 20 μ L by using 4 U/ μ L Superscript II (Gibco BRL), 1 U/ μ L RNasin (Promega, Madison, Wis), 2 μ mol/L specific reverse transcription primer (IL-4, 5'-TGG TTG GCT TCC-3'; IFN- γ , 5'-CAG TTC AGC CAT-3'; and β -actin, 5'-GTG GAC GAT GGA-3'), and 0.5 mmol/L of each dNTP (Takara Biomedicals, Ohtsu, Japan) in RT buffer (50 mmol/L Tris-HCl, 75 mmol/L KCl, 3 mmol/L MgCl₂, and 10 mmol/L dithiothreitol) at 42°C for 2 hours, respectively. The reaction mixtures were heated at 95°C for 5 minutes to terminate the action of the reverse transcriptase.

Conjugation of fluorescent dye to oligonucleotide primer

Fluorescent dye labeling of PCR primers was performed according to the method described elsewhere.²⁸

PCR

A 4- μ L aliquot of the reverse transcription product was added to 20 μ L of PCR reaction mixture containing 10 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.001% gelatin, 0.5 U of Taq polymerase (Perkin Elmer, Foster City, Calif), 0.5 μ mol/L Rhodamine-labeled antisense primer (IL-4, 5'-rhodamine X isothiocyanate (XRITC) TCT CAG TTG TGT TCT TGG AGG-3'; IFN- γ , 5'-(XRITC) ACA TTC AAG TCA GTT ACC GA-3'; and β -actin, 5'-(XRITC) ACT CGT CAT ACT CTT GCT TG-3'), and 0.5 μ mol/L sense primer (IL-4, 5'-ATG TGC CCG GGA ACT TTG TC-3'; IFN- γ , 5'-CAG GTC ATT CAG ATG TAG CG-3'; and β -actin, 5'-TGA AGT CTG ACG TGG ACA TC-3').²⁹ PCR was performed in a thermal cycler 9600R (Perkin Elmer) after an initial denaturation at 94°C for 1 minute. Each thermal cycle consisted of denaturation for 1 minute at 94°C, annealing for 2 minutes at 65°C, and extension for 2 minutes at 72°C. The final extension was at 72°C for 7 minutes. Amplification was repeated for 31 cycles for IL-4, 25 cycles for IFN- γ , and 20 cycles for β -actin and resulted in generation of PCR products of 149 bp, 265 bp, or 246 bp in size, respectively. All reactions were performed in triplicate.

Synthesis of standard RNA

The cDNA fragments for standard RNA synthesis were cloned into pT7 Blue T-vector (Novagen, Madison, Wis) by means of the

RT-PCR technique, with each set of primers as follows: IL-4, sense primer 5'-ATG GGT CTC ACC TCC CAA CTG CT-3' and antisense primer 5'-CGA ACA CTT TGA ATA TTT CTC TCT CAT-3'; IFN- γ , sense primer 5'-ATG AAA TAT ACA AGT TAT ATC TTG GCT TT-3' and antisense primer 5'-GAT GCT CTT CGA CCT CGA AAC AGC AT-3'; and β -actin, sense primer 5'-GTG CGT GAC ATT AAG GAG AAG C-3' and antisense primer 5'-CAT TTG CGG TGG ACG ATG GAG-3'. The sizes of cDNA fragments cloned were 456 bp for IL-4, 494 bp for IFN- γ , and 497 bp for β -actin.³⁰ The cDNA-containing plasmids were linearized with *Ava* I for IL-4 or *Eco* R I for IFN- γ and β -actin, purified, and subjected to *in vitro* transcription to generate complementary RNA (cRNA) by using T7 RNA polymerase (Ambion, Inc, Austin, Tex), according to the manufacturer's instructions. Specificity of the cDNA fragments for standard RNA synthesis and the RT-PCR products was confirmed by Southern blotting and hybridization with an internal oligonucleotide probe and digestive analysis with the following restriction enzymes: *Eco* RV for IL-4, *Dra* I for IFN- γ , and *Sca* I for β -actin.

The contaminating DNA was removed by extensive digestion with RNase-free DNase I. The resulting cRNA was quantitated by spectrophotometry and stored in 70% ethanol at -80°C until use.

PCR product quantitation

Rhodamine-labeled PCR products were analyzed in a fluorescence-bioimage analyzer, which measured laser-induced fluorescence. The PCR product was diluted with 40 μ L of 0.5 $\times$ Tris, boric acid, Na₃ EDTA (TBE) buffer in the same tube in which PCR was performed. Three microliters of the diluted PCR product was mixed with 1 μ L of 4 $\times$ loading buffer (25% glycerol, 5 mmol/L EDTA, and 0.4% crystal violet) and electrophoresed on 6% polyacrylamide gel in 0.5 $\times$ TBE buffer. The gel was then scanned with a green laser (532 nm) in the fluorescence-bioimage analyzer FMBIO-100 (Hitachi Software Engineering Co, Ltd, Yokohama, Japan).^{31,32} The digitalized imaging data were analyzed by using the FMBIO image analysis program (Hitachi Software Engineering Co, Ltd). The amount of mRNA in each PBMC sample was determined by comparison of signal density with that of a standard curve generated from simultaneously amplified serial dilutions of standard RNA, which were adjusted relative to the corresponding β -actin signal to normalize for RNA levels used in the PCR reaction. Values represent the mean of 3 parallel PCR amplifications. The intra-assay (lane-to-lane) coefficient of variation for the quantitation of identical samples averaged 16.9%.

Data analysis

The data obtained were analyzed with the 2-tailed Student's *t* test for comparison with the control data. Any *P* value less than .05 was considered significant.

RESULTS

Biphasic effect of IL-12 on Df-induced IL-4 production by PBMCs from patients with mite-sensitive BA

As shown previously, a 1 μ g/mL dose of Df was capable of stimulating maximum production of IL-4 in patients with active BA, and cells cultured for 2 days induced the maximal response. These numbers of IL-4-producing cells were approximately 3- to 5-fold higher in patients with active asthma relative to the numbers observed in cultures of cells from nonasthmatic individuals (Fig 1). The generation of IL-4-secreting cells by

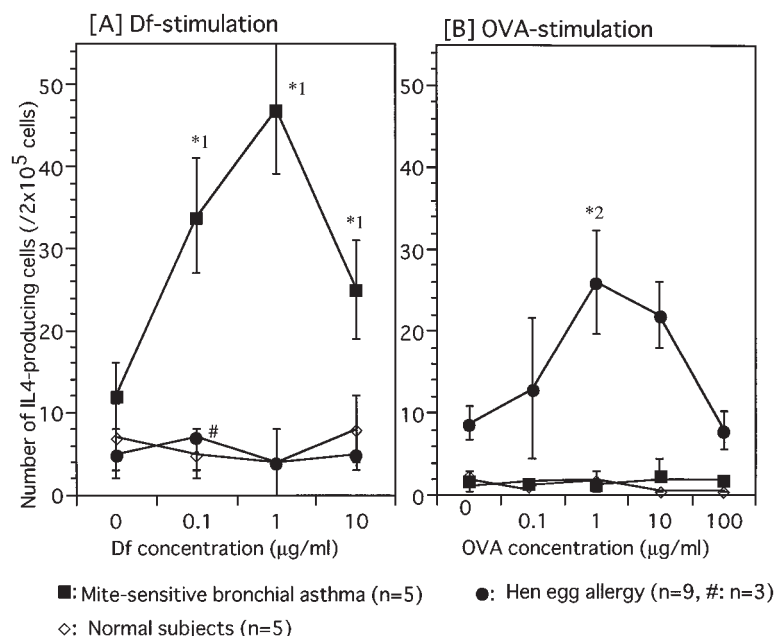


FIG 1. Df-specific induction of IL-4-secreting cells by mite-sensitive BA. Mononuclear cells (1×10^6) were added to 1 µg/mL Df antigen in 1 mL of RPMI medium supplemented with 10% heat-inactivated pooled human serum and cultured for 2 days at 37°C in a 10% CO₂ atmosphere. An ELISPOT assay was performed to evaluate the ability of these cells to produce IL-4. Spots representing single IL-4-secreting cells that developed during 4 hours of incubation at 37°C were counted with an inverted microscope. The specificity of the ELISPOT assay for detecting IL-4-secreting cells was determined by inhibition with a soluble antibody to IL-4. *1, $P < .05$ compared with normal individuals and patients with allergy to hen eggs; *2, $.01 < P < .02$ compared with normal individuals.

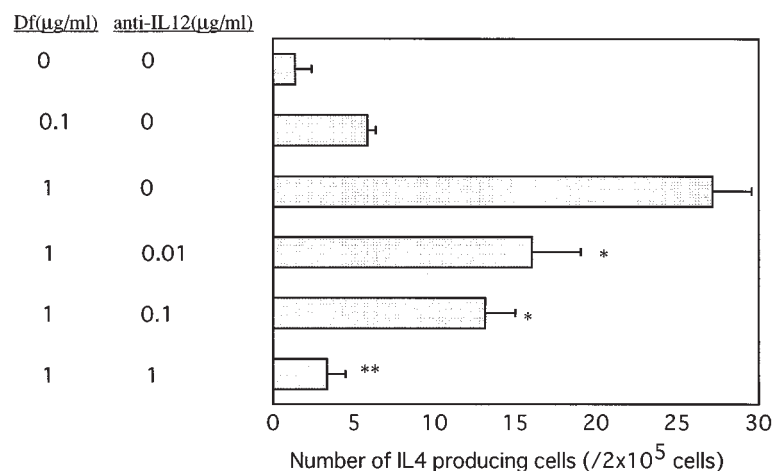


FIG 2. IL-12 is required for Df-induced IL-4 production. To evaluate the requirement for endogenous IL-12 for Df-specific IL-4 production by mononuclear cells from patients with mite-sensitive BA, Df-stimulated cells were added to serially diluted anti-IL-12 antibody and then cultured for 48 hours to induce IL-4-secreting cells. An ELISPOT assay was performed, as shown in Fig 1. Data represent means $\pm$ SEM of 6 independent experiments with 6 different subjects. * $P < .01$ compared with untreated cells; ** $P < .001$.

Df-stimulated lymphocytes was specific for relevant antigen (Fig 1). Addition of anti-IL-12 antibody to the cultures blocked Df-induced IL-4 production in a dose-dependent fashion (Fig 2). Thus endogenous IL-12 is required for Df-induced IL-4 production. To determine the direct effect of IL-12 on Df-induced IL-4 production, Df-stimulated patient PBMCs were added to serial dilu-

tions of IL-12. High doses (1 to 10 ng/mL) of IL-12 were capable of enhancing Df-induced IL-4 production, whereas low doses (0.05 to 0.1 ng/mL) of IL-12 down-regulated IL-4 production (Fig 3). These phenomenon were not observed in Df-stimulated control PBMCs (data not shown).

In contrast, patient PBMCs were cultured with 1

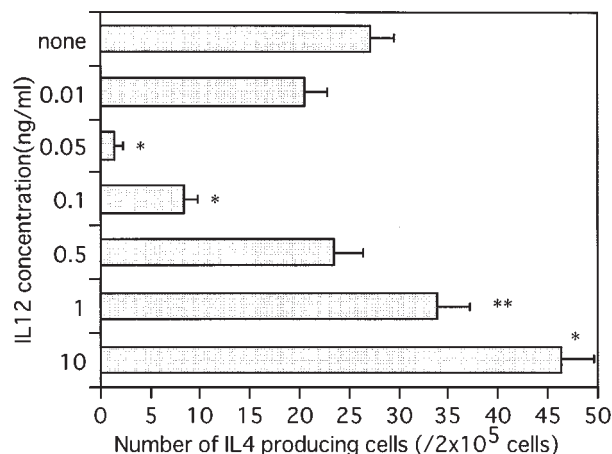


FIG 3. Effect of IL-12 on Df-induced IL-4 production. To evaluate the effect of IL-12 on Df-induced IL-4 production, mononuclear cells from patients with mite-sensitive BA were added to serially diluted recombinant IL-12 simultaneously on stimulation with a 1 μ g/mL dose of Df antigen and then cultured 48 hours for IL-4 production. An ELISPOT assay was performed to evaluate the ability of these cells to produce IL-4, as shown in Fig 1. Data represent means $\pm$ SEM of 3 independent experiments with 3 different subjects. * P < .05 compared with untreated cells; ** P < .001 compared with untreated cells.

μ g/mL Df for 3 days, after which IFN- γ activity in the culture supernatant was measured by ELISA. As shown previously, IFN- γ production by Df-stimulated patient PBMCs decreased in an antigen-specific manner in comparison with unstimulated PBMCs (Fig 4). Addition of IL-12 to the cultures enhanced Df-induced IFN- γ production in a dose-dependent fashion (Fig 4), whereas cells cultured for 5 days induced the maximal response. Thus IL-12 was capable of increasing Df-induced IFN- γ production.

Effect of IL-12 on Df-induced mRNA for IL-4 production

To verify the increasing effect of IL-12 on Df-induced IL-4 production at messenger levels, expression of mRNA by IL-12-treated cells on stimulation with Df antigen was quantified by laser-induced fluorescence analysis of RT-PCR-amplified DNA fragments after size fractionation through PAGE. Standard RNA was serially diluted (10^3 to 10^9 copies per tube) and amplified by RT-PCR in triplicate. As shown in Fig 5, A, we observed a linear increase of the fluorescence signal with the RNA template input over a range of 2.0×10^3 copies to 1.2×10^8 copies of standard RNA per 20 μ L of RT reaction. Results in Fig 5, B, and Table I are expressed as the copy number of IL-4 or IFN- γ per 0.5 μ g of total RNA obtained from 1×10^6 cultured cells with reference to standard RNA.

The fluorescence signal of IL-4 mRNA in unstimulated cells from patients was 3.1×10^4 copies per 0.5 μ g total RNA, whereas on Df stimulation, this mRNA level was increased (Fig 5, B, and Table I). On treatment with a low dose of IL-12 (0.05 ng/mL), the signal was decreased, but it was increased on treatment with a high dose of IL-12 (1 and 10 ng/mL). These results indicate

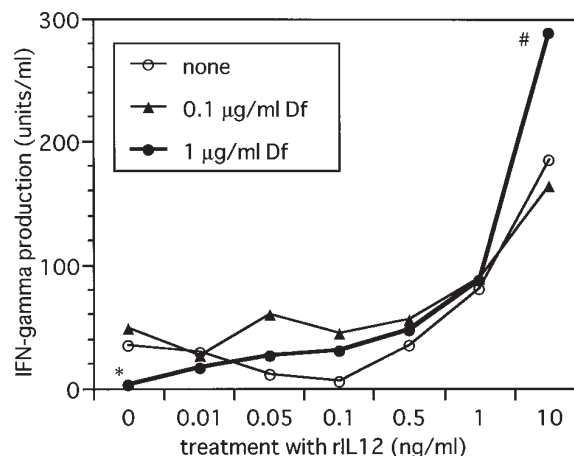


FIG 4. Effect of IL-12 on Df-induced IFN- γ production in patients with BA. To examine the effect of IL-12 on Df-induced IFN- γ production, mononuclear cells (1×10^6) from patients with mite-sensitive BA were added to serially diluted recombinant IL-12 simultaneously with stimulation by 0.1 or 1 μ g/mL doses of Df antigen in 1 mL of RPMI medium supplemented with 2% heat-inactivated pooled human serum and then cultured for 3 days at 37°C in a 10% CO₂ atmosphere. Culture supernatants from the cells were harvested and assayed for IFN- γ activity with a solid-phase ELISA. Results are expressed as units of IFN- γ per milliliter of supernatant referenced to a standard IFN- γ . Data are expressed as the mean of triplicate trials in a representative experiment of 4 independent experiments with 4 different subjects. * P < .01 compared with unstimulated cells; # P < .001 compared with IL-12-untreated cells.

that at high doses IL-12 is capable of augmenting mRNA expression for IL-4 protein synthesis (although low doses of IL-12 inhibit the expression). These phenomena observed at the mRNA level were similar to those

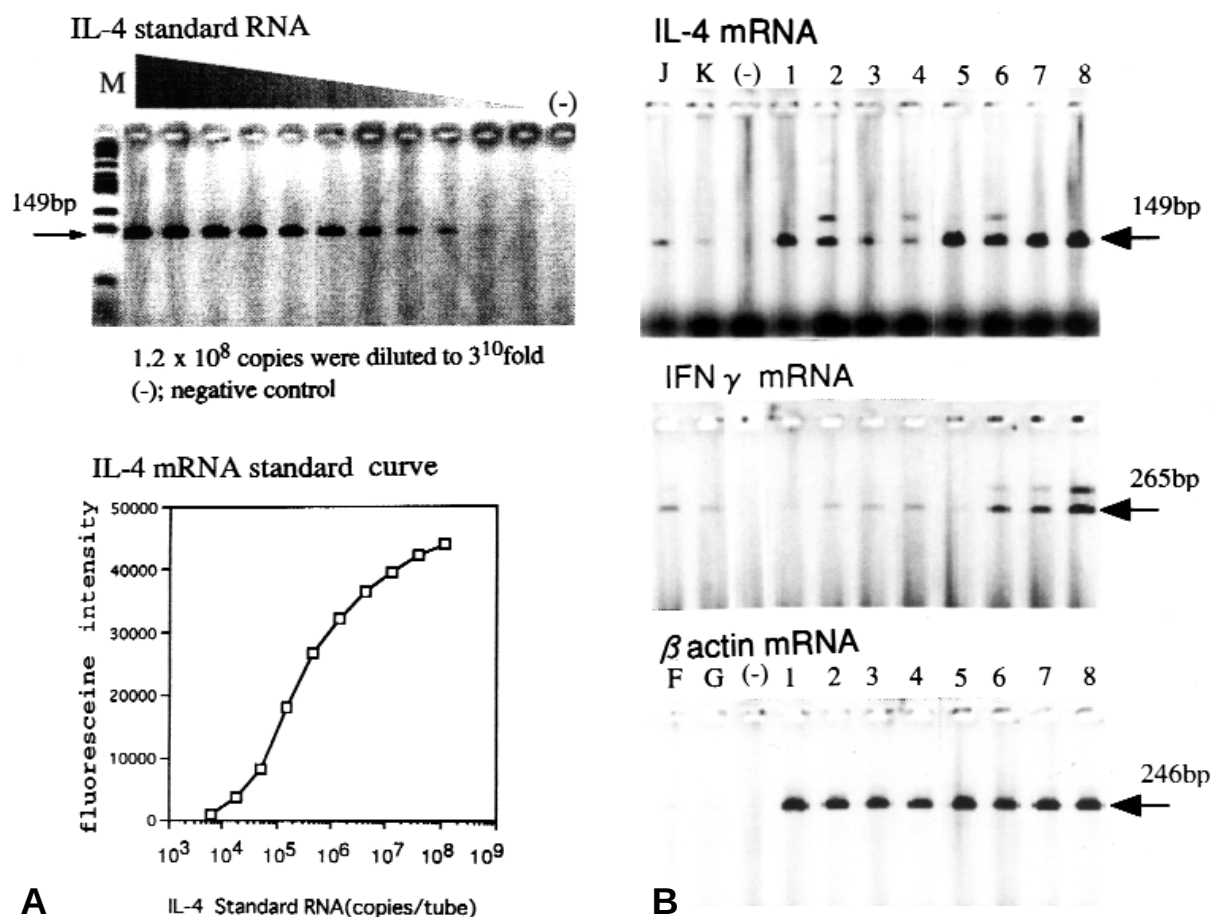


FIG 5. Effect of IL-12 on Df-induced mRNA expression of IL-4 and IFN- γ production. To verify the increasing effect of IL-12 on Df-induced IL-4 and IFN- γ production at messenger levels, patient PBMCs were added to 0.05 or 10 ng/mL recombinant IL-12 simultaneously with stimulation by 1 μ g/mL of Df antigen and then cultured for 48 hours. The amount of mRNA for IL-4 and IFN- γ in each PBMC sample was quantified by analysis of RT-PCR-amplified DNA fragments by laser-induced fluorescence. Results are expressed as copy number of IL-4 or IFN- γ per 0.5 μ g of total RNA obtained from 1 × 10⁶ cultured cells. **A**, Standard RNA was diluted serially and amplified by RT-PCR in triplicate. A linear increase of the fluorescence signal with the RNA template input was observed over a range of 2.0 × 10³ copies to 1.2 × 10⁸ copies of standard RNA per 20 μ L of RT reaction. **B**, The fluorescence signal of mRNA for IL-4 in unstimulated cells from patients was 3.1 × 10⁴ copies per 0.5 μ g total RNA (lane 1), whereas signals were increased on Df stimulation (lane 5). On treatment with a low dose (0.05 ng/mL) of IL-12 (lane 6), the extent of the signals was decreased, whereas treatment with a high dose (1 and 10 ng/mL) of IL-12 (lanes 7 and 8) increased the signal. In contrast, the signals of mRNA for IFN- γ protein synthesis were increased on Df stimulation in a dose-dependent fashion (lanes 1 to 4 and 5 to 8). Data are expressed as a representative experiment of 3 independent experiments with 3 different subjects. Lanes F, G, J, and K: standard RNA; (-): negative controls; lanes 1 to 8: refer to Table I.

observed in Df-induced IL-4 protein secretion. In contrast, the signals of mRNA for IFN- γ protein synthesis were increased on Df stimulation in a dose-dependent fashion (Fig 5, B, and Table I). The total copy number of IL-4 or IFN- γ mRNA amplified by RT-PCR in total RNA from the cultured cells per tube was similar to that found in 0.5 μ g of total RNA (data not shown). The copy number of β -actin mRNA in each sample was amplified by RT-PCR (Fig 5, B), and the results per β -actin were similar to those in 0.5 μ g of total RNA (data not shown).

Effect of IL-12 on Df-induced IgE production by PBMCs from patients with mite-sensitive BA

Because previous studies^{33,34} clearly indicated that IL-4 is an essential factor for IgE synthesis, we investigated whether the effect of IL-12 on IL-4 production is attributable to the IgE synthesis. The results presented in Fig 6 show that IL-12 suppressed Df-induced IgE production at low doses (0.01, 0.05, and 0.1 ng/mL), whereas at high doses (10 ng/mL), IL-12 increased production of the same.

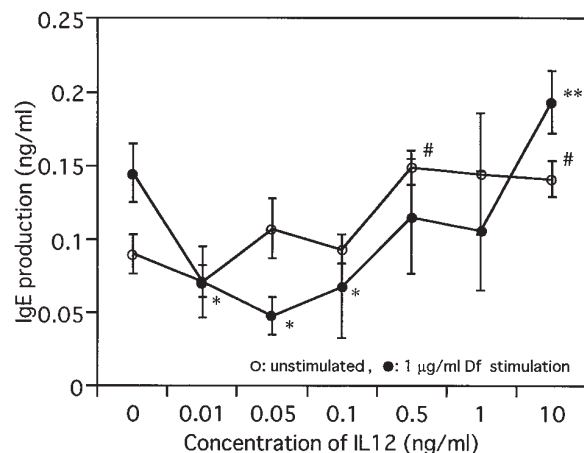


FIG 6. Effect of IL-12 on Df-induced IgE production. To examine the effect of IL-12 on Df-induced IgE production, patient PBMCs (1×10^6) were added to serially diluted recombinant IL-12 simultaneously with stimulation by $1 \mu\text{g/mL}$ Df antigen and then cultured for 9 days at 37°C in a $10\% \text{CO}_2$ atmosphere in 1 mL of RPMI medium supplemented with 10% heat-inactivated pooled human serum. Total IgE activity for the culture supernatants was measured by solid-phase ELISA. Data are expressed as means $\pm$ SEM of 3 independent experiments with 3 different subjects. #, $*P < .01$; $**P < .05$ as compared with untreated cells.

TABLE I. The effect of IL-12 treatment on expression of IL-4 or IFN- γ mRNA

Patient no	Df (1 $\mu\text{g/mL}$)	Treatment	IL-4 mRNA ($\times 10^4$)	IFN- γ mRNA ($\times 10^4$)
1	—	None	3.1	0.3
2	—	0.05 ng/mL IL-12	2.1*	1.5*
3	—	1 ng/mL IL-12	0.6*	2.1*
4	—	10 ng/mL IL-12	0.7*	2.6*
5	+	None	6.4*	0.8*
6	+	0.05 ng/mL IL-12	2.9†	17†
7	+	1 ng/mL IL-12	8.0†	20†
8	+	10 ng/mL IL-12	9.8†	100†

Data show the mean of mRNA copies per $0.5 \mu\text{g}$ total RNA in triplicate assays in a representative experiment (see legend for Fig 5).

* $p < .05$ compared with patient 1.

† $p < .05$ compared with patient 5.

To verify this phenomenon in Df-specific IgE production, Df-specific IgE in serum from SCID mice reconstituted with PBMCs from a patient with Df-sensitive BA was measured with an ELISA. Mice reconstituted with unstimulated patient PBMCs significantly produced Df-specific IgE 1 week after reconstitution (Fig 7, A). Mice transferred Df-pulsed patient cells also produced similar levels of specific IgE at that time, and the activity increased significantly 3 weeks after reconstitution as compared with unstimulated cells; afterwards the IgE production decreased gradually until the sixth week (Fig 7, A). To verify the specificity of IgE production, the sera were treated 3 times in a 96-well, flat-bottom plate coated with anti-IgE mAb (from Yamasa in Japan, differed

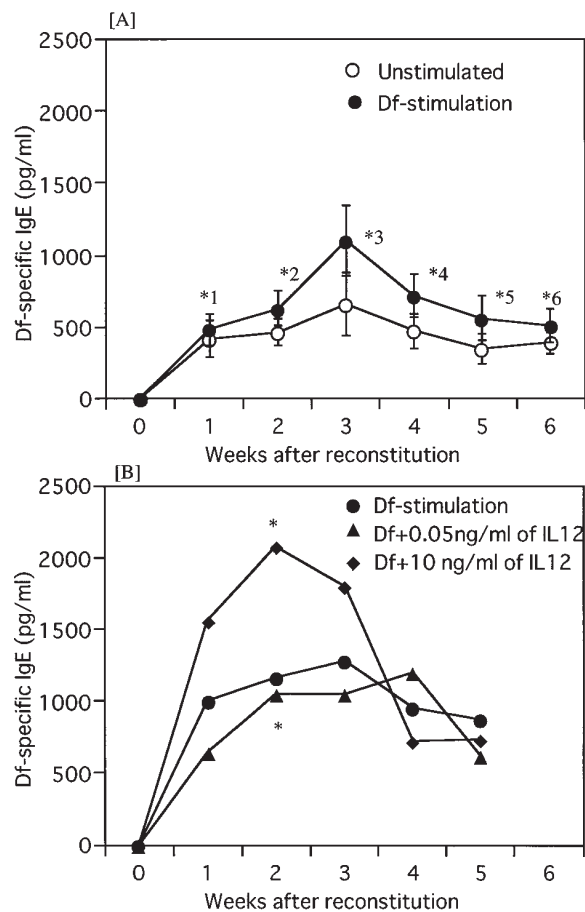


FIG 7. Effect of IL-12 on Df-specific IgE production and Df-specific IgE production in SCID mice reconstituted with human PBMCs. **A**, Patient PBMCs (1×10^7) were suspended in the presence of $10 \mu\text{g/mL}$ Df antigen in 4 mL of serum-free RPMI-1640 medium in vitro. After 4 hours' culture at 37°C in a $10\% \text{CO}_2$ atmosphere, the cells were washed 4 times with PBS and then injected intraperitoneally in $500 \mu\text{L}$ of PBS into SCID mice. On the day indicated in the Results section, the mice sera were collected, and Df-specific IgE activity of the serum was measured with a solid-phase ELISA by using plates coated with Df at $50 \mu\text{g/mL}$. Data represent means $\pm$ SEM of 6 independent experiments with 6 different subjects. *1, $P = .305$; *2, $P = .035$; *3, $P = .005$; *4, $P = .029$; *5, $P = .030$; *6, $P = .159$ compared with unstimulated cells. **B**, To examine the effect of IL-12 on Df-induced specific IgE production, the Df-stimulated cells were treated with 0.05 or 10 ng/mL of recombinant IL-12 in vitro. The cells were injected intraperitoneally into SCID mice, and Df-specific IgE activity of the serum was measured as shown in **A**. A representative datum from 3 independent experiments is shown because the data varied, although the results were similar. * $P < .001$ compared with IL-12-untreated cells.

from the antibody used in the ELISA) or Df antigen for 3 hours at room temperature. The remaining IgE activity of the serum treated was evaluated with ELISA. The IgE activity decreased in both sera compared with untreated sera (percent inhibition, approximately 90%). In contrast, sera treated with anti-IgG polyclonal antibody did not show decreased activity (data not shown). The combined data indicate that serum obtained from Df-pulsed

PBMC-transferred SCID mice is specific for both IgE and Df antigen.

To evaluate the effect of IL-12 on Df-specific IgE production, Df-pulsed patient PBMCs were treated with IL-12 and then were transferred to SCID mice. A 0.05 ng/mL dose of IL-12 suppressed the Df-specific IgE production, whereas a 10 ng/mL dose of IL-12 was capable of increasing the production (Fig 7, B).

DISCUSSION

In neonatal cells both T_{H2} -type and T_{H1} -type cells were activated when the cells were exposed to IL-12.¹⁸ This process may cause susceptibility to infection and inoculation early in life and increases the side effects seen in infection.³⁵⁻³⁷ In contrast to the susceptibility, the initiation and augmentation of IL-4 production in the activated cord blood T cells^{18,38} implies that infants exposed to an allergen, such as Df antigen, may have allergic diseases mediated by IL-12 secreted by infected cells, such as phagocytic cells, NK cells, and T cells,^{7,8,9} when infected recurrently with some microorganism early in life. In this study we investigated the influence of IL-12 on Df antigen-induced T_{H2} - or T_{H0} -type cell activation, especially IL-4 secretion, and subsequent IgE production by using PBMCs from young patients with Df antigen-sensitized BA. Indeed, the high-dose IL-12 induced IL-4 production by Df-activated PBMCs from pediatric patients allergic to mite antigen, which is similar to the increase in IL-4 production observed in neonates.^{18,38} This phenomenon was verified by the results of mRNA analysis with a fluorescence-bioimage analyzer measuring laser-induced fluorescence of rhodamine-labeled PCR products³² and by subsequently induced IgE production by the patient cells. These indicated that at a low dose of IL-12, IL-4 and IFN- γ production are regulated reciprocally, and that at high doses of IL-12, cells produced IL-4 and IFN- γ simultaneously, and both cytokines were unregulated. In vivo high doses of IL-12 produced by infected cells are likely to induce both T_{H1} - and T_{H2} -type cells or T_{H0} -type cell activation in privileged sites of infection, and consequently the IL-4 production may overcome T_{H1} -type cell activation.

In contrast, it is reported that addition of a high dose (10 ng/mL) of IL-12 at time periods after initial antigen stimulation resulted in less inhibition of IL-4 production by allergic donor-derived CD4⁺ T cells, which were activated with PHA and phorbol myristate acetate before IL-4 production.¹⁷ However, it was clearly demonstrated that IL-12 can stimulate IL-4 production in recall, but not primary, immune responses without stimulation of mitogen, including PHA and phorbol myristate acetate in this study, or in activated neonatal cells.^{18,38} The difference in experimental procedures may reconcile the previous report.¹⁷ IL-12 may initiate the disease process or enhance the susceptibility already initiated in genetically susceptible individuals at the sites of infection.

If these in vitro observations are applicable clinically, infants exposed to an allergen acquired the nature to

develop an allergy, such as BA, early in life when recurrently infected with microorganisms that induced IL-12 production by such primitive cells as phagocytic cells. We demonstrated that endogenous IL-12 is required for Df-specific IL-4 production by mononuclear cells, whereas exogenous IL-12 had a different effect on antigen-induced IL-4 and IFN- γ production depending on whether low-dose or high-dose IL-12 is added to the cultures. Therefore extremely low levels of IL-12 (<0.05 ng/mL) might be induced by allergen-stimulated mononuclear cells, and IL-12, produced by infected cells, likely modulates the amount of IL-4 produced by allergen-activated mononuclear cells.

RSV infection is an infectious disease of the respiratory tract seen in neonates.³⁹⁻⁴¹ Because some patients experience recurrent wheezing after RSV infection, attention has been directed to progression of the disease to BA.⁴² After RSV infection, lymphocytes acquire specific susceptibility to Df and food antigens, particularly ovalbumin, probably by secreting such cytokines as IL-4, which is capable of inducing T_{H2} - or T_{H0} -type cells.⁴³ Activation of T_{H1} , T_{H2} , or T_{H0} cells^{44,45} involved in the immune response in allergic reactions is likely to be induced in lymphocytes after RSV infection, resulting in an increase in secretion of cytokines (eg, IL-2, IL-3, GM-CSF, IL-4, IL-5, IL-6, and IFN- γ), with a consequent imbalance⁴⁶ being likely. Production of IL-12 by RSV-infected cells may involve the production of these cytokines because high, but not low, doses of IL-12 are capable of inducing IL-4 and IgE production in Df-activated lymphocytes from children with mite-sensitive BA, as shown in this study. If lymphocytes from infants disposed to allergy whose lymphocytes were activated by allergen are infected with a virus such as RSV, high doses of IL-12 produced by RSV-infected cells may cause atopic disease, particularly after recurrent exposures to allergens. Thus we showed that in pediatric patients with asthma, low-dose and high-dose IL-12 induce T_{H1} responses, and high-dose IL-12 induces both T_{H1} responses and T_{H2} or T_{H0} responses. Consequently, the IL-4 production may overcome T_{H1} -type cell activation of IgE production. Given further study, we should be able to elucidate the phenomena that IL-12 levels after infection, including RSV infection in infants in vivo, are comparably high, as demonstrated in vitro by the ability to support the development of a T_{H2} or T_{H0} phenotype.

We believe the present observations shed light on the pathogenesis, and potentially the prevention, of allergic diseases in infants.

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