

Predominance of type 2 cytokine-producing CD4⁺ and CD8⁺ cells in patients with atopic dermatitis

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Background: Recently, increased IL-4 and decreased interferon- γ (IFN- γ) production in peripheral blood mononuclear cells (PBMCs) from patients with atopic dermatitis have been reported by several groups. They measured the total amount of each cytokine in culture supernatants in their studies. These studies suggested a predominance of type 2 cytokine-producing cells in atopic dermatitis. However, it is still unclear whether the cytokine imbalance is the result of an imbalance of specific T-cell subsets or of dysregulation of the cytokine-producing ability in T-cell subsets. Here, we examined frequencies of IL-4⁺, IFN- γ ⁺, or IL-2-producing CD4⁺ cells and CD8⁺ cells in PBMCs from patients with atopic dermatitis at a single cell level by using flow cytometry.

Methods: PBMCs from 45 patients with atopic dermatitis and 24 healthy control subjects were stimulated with immobilized anti-CD3 monoclonal antibody for 6 hours in the presence of monensin. Cells were fixed, made permeable, and stained for intracellular cytokines in combination with staining for cell surface markers CD4 and CD8.

Results: The frequency of IL-4-producing CD4⁺ cells and CD8⁺ cells from patients with atopic dermatitis was significantly higher ($p < 0.001$) than that from healthy control subjects. In contrast, the frequency of IFN- γ -producing CD4⁺ cells and CD8⁺ cells was significantly decreased ($p < 0.01$) in the patients with atopic dermatitis. The frequency of IL-2-producing cells from patients with atopic dermatitis was comparable to that from healthy control subjects.

Conclusion: Our findings indicate that frequencies of type 2 cytokine-producing cells, not only among CD4⁺ cells but also among CD8⁺ cells, were significantly higher in patients with atopic dermatitis than in healthy control subjects. (*J Allergy Clin Immunol* 1997;99:673-82.)

Key words: Atopic dermatitis, IL-4, interferon- γ , IL-2, CD4⁺ cells, CD8⁺ cells, flow cytometry

Abbreviations used

AD:	Atopic dermatitis
FITC:	Fluorescein isothiocyanate
HEPES:	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
IFN- γ :	Interferon- γ
mAb:	Monoclonal antibody
PBMCs:	Peripheral blood mononuclear cells
PBS:	Phosphate-buffered saline
PE:	Phycoerythrin
PerCP:	Peridinin chlorophyll

Profiles of cytokine secretion have important roles in the immune response and the susceptibility to diseases. CD4⁺ T helper (T_H) cells are categorized into at least two distinct subsets on the basis of their profiles of cytokine production: T_{H1} cells, which produce type 1 cytokines such as IL-2, interferon- γ (IFN- γ), and lymphotoxin and T_{H2} cells, which secrete type 2 cytokines, IL-4, IL-5, IL-6, IL-10, and IL-13, were originally identified among a panel of mouse helper T-cell clones.^{1,2} T_{H1}-like and T_{H2}-like patterns have also been identified among human T_H cell clones.^{3,4} Recent studies have shown that cytokine production is not limited to CD4⁺ T cells. Stimulated mouse normal CD8⁺ T cells usually secrete type 1 cytokines.^{5,6} However, some human CD8⁺ T-cell clones secrete both type 1 and type 2 cytokines.^{7,8} Furthermore, several investigators have reported that type 1 or type 2 cytokines are produced by CD8⁺ T-cell clones from patients with leprosy and that some of type 2 cytokine-secreting CD8⁺ T-cell clones have been reported to suppress the proliferation of *Mycobacterium leprae*-specific CD4⁺ T cell (T_{H1}) clones.⁹ Cytotoxic CD8⁺ T-cell clones producing IL-4 but not IFN- γ were isolated from HIV-infected patients with a syndrome resembling Job's syndrome.¹⁰ The effect of stimulation with IL-4 to polarize CD4⁺ T cells into T_{H2} cells is well known.^{11,12} Also, naive murine CD8⁺ T cells were polarized into type 2 cytokine-producing cells when they were stimulated with IL-4.¹³⁻¹⁵ Functionally, type 1 cytokine-producing cells promote cell-mediated immune responses, such as delayed-type hypersensitivity reactions and macrophage activity. In contrast, type 2 cytokine-producing cells promote hu-

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moral immune responses, helping IgE switching and secretion from B cells and eosinophil activation.

Atopic dermatitis (AD) is a chronic inflammatory skin disease frequently associated with markedly elevated IgE levels,¹⁶⁻¹⁸ and recent studies indicate that this elevation is the result of T-cell dysregulation.^{19, 20} Because T cells are potent producers of a large variety of cytokines, studies have been undertaken to investigate cytokine production in atopic diseases. On polyclonal stimulation, peripheral blood mononuclear cells (PBMCs) from atopic patients produce lower amounts of IFN- γ ²¹⁻²⁴ and higher amounts of IL-4²²⁻²⁵ compared with PBMCs from nonatopic healthy control subjects. These results suggest that expansion or upregulation of type 2 cytokine-producing cells occurs in atopic patients.

Recently, flow cytometric analysis of cytokine-producing cells has been developed.^{26, 27} In this study we used this method to detect intracellular cytokines and analyzed the frequencies of IL-4-, IFN- γ -, or IL-2-producing cells in either CD4⁺ or CD8⁺ cells from patients with AD. The role of these cells in AD is also discussed.

METHODS

Subjects

Peripheral blood was obtained from 45 patients with AD (19 men and 26 women, aged 25.4 ± 8.6 years [mean $\pm$ SD]). AD was diagnosed according to the criteria of Hanifin and Rajka.²⁸ The severity of disease was determined by the criteria of Rajka and Langeland,²⁹ and patients were categorized into three groups. Briefly, symptoms of AD were scored (1 to 3 points in each parameter) by using the following parameters: (1) extent, (2) course (by history), and (3) intensity (disturbance of night's sleep by itching). Mild, moderate, or severe AD was determined by score summation. Patients with AD were treated, depending on the severity of the disease, with betamethasone valerate, hydrocortisone butyrate, or buprenorphine. Control blood samples were also obtained from 24 nonatopic healthy volunteers (11 men and 13 women, aged 33.1 ± 6.2 years). Patients with AD were selected at the Department of Dermatology, Yokohama City University Hospital. Informed consent was obtained from all subjects before these studies were performed.

Monoclonal antibodies

Anti-human IL-2 and anti-IL-4 monoclonal antibodies (mAbs) (mouse IgG₁) were purchased from Genzyme (Cambridge, Mass.). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG₁ antibody was purchased from Southern Biotechnology Associates, Inc. (Birmingham, Ala.). Phycoerythrin (PE)-conjugated anti-human IFN- γ (mouse IgG₁) was purchased from PharMingen (San Diego, Calif.). FITC- or PE-conjugated anti-CD4 mAb and peridinin chlorophyll (PerCP)-conjugated anti-CD8 mAb were purchased from Becton Dickinson (San Jose, Calif.). PE-labeled, subclass-matched control Ig was purchased from Becton Dickinson. Anti-CD3 mAb was isolated from culture supernatants of OKT3 cell line (American Type Culture collection, Rockville, Md.) by using protein G Sepharose (PharMingen).

Cell cultures

PBMCs were isolated from heparinized blood by Ficoll (HISTOPAQUE-1077; Sigma, St. Louis, Mo.) density centrif-

ugation and washed three times with RPMI-1640 (Gibco BRL, Grand Island, N.Y.). PBMCs (4×10^6 cells/ml) were cultured in 24-well culture plates (Sumitomo Bakelite Co., Ltd, Tokyo, Japan) in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (Biological Industries, Haemek, Israel), 100 U/ml penicillin (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan), 100 mg/ml streptomycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan), 30 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer (Wako Junyaku Co., Ltd., Osaka, Japan), 2×10^{-5} mol/L 2-mercaptoethanol (Sigma), and 2 mmol/L L-glutamine (Wako Junyaku Co., Ltd.). Cells were cultured for 6 to 24 hours at 37°C in 5% CO₂ in an incubator with 85% humidity. These cells were stimulated with immobilized anti-CD3 mAb in the presence or absence of monensin (1 μ mol/L) (Sigma) for the last 6 hours.

Staining

For immunostaining of cytoplasmic IL-2 and IL-4, cells were washed twice in phosphate-buffered saline (PBS) and then fixed in cold PBS containing 2% paraformaldehyde for 15 minutes. After two additional washes in PBS, cells were resuspended to a concentration of 1×10^6 in 100 μ l of PBS containing 0.1% saponin, 10% human AB blood type serum, and 1 μ g/ml goat IgG (Chemicon International Inc., Temecula, Calif.). After 10 minutes, cytokine-specific mAb was added at a concentration of 1 μ g/ml for 30 minutes on ice. The cells were washed twice in PBS containing 0.1% saponin (saponin buffer) and resuspended in 100 μ l of saponin buffer with 1 μ g/ml goat IgG and 1.5 μ g/ml FITC-conjugated goat anti-mouse IgG₁ for 30 minutes in the dark at room temperature. Cells were washed once in saponin buffer, followed by two additional washes in PBS, and then resuspended in 100 μ l of PBS containing 2 μ g/ml mouse IgG (Chemicon International Inc.) for 10 minutes on ice. PE-conjugated anti-CD4 mAb and PerCP-conjugated anti-CD8 mAb were added for 30 minutes on ice. Finally, the cells were washed twice in PBS and resuspended in approximately 500 μ l of PBS. For the negative control, cells were stained with FITC-conjugated second antibody without anti-cytokine mAbs, followed by staining with anti-CD4 mAb and anti-CD8 mAb.

Immunostaining of cytoplasmic IFN- γ was performed as follows. Cultured or freshly isolated PBMCs were washed twice in PBS, and FITC-conjugated anti-CD4 mAb and PerCP-conjugated anti-CD8 mAb were added for 30 minutes on ice. Cells were washed twice in PBS and then fixed with 4% paraformaldehyde for 20 minutes on ice. After two additional washes in PBS, the cells were made permeable with saponin buffer, and PE-conjugated anti-IFN- γ mAb was added at a concentration of 1 μ g/ml for 30 minutes on ice. For the negative control, cells were stained with PE-conjugated mouse IgG₁ for 30 minutes on ice. Cells were washed twice in saponin buffer and once in PBS and then resuspended in approximately 500 μ l of PBS.

To verify the specificity of the staining procedure, we confirmed that the binding of each mAb was blocked with an excess of recombinant cytokine (IL-2, IL-4, or IFN- γ [PharMingen]).

Flow cytometry

Cells were analyzed on a FACScan flow cytometer (Becton Dickinson) equipped with a 15 mA argon ion laser; and filter settings for FITC (BP 530/30), PE (BP 585/42), and PerCP (LP 650) were used. Ten thousand cells were acquired into the list mode, and the data were analyzed with CELLQuest software (Becton Dickinson). Analysis gates were set on lymphocytes

according to forward- and side-scatter properties. Results were expressed as the percentage of cytokine-producing cells in each CD4⁺ or CD8⁺ cell population.

Statistical studies

Statistical studies were performed by using the unpaired Student's *t* test.

RESULTS

Frequency of CD4⁺ and CD8⁺ cells from patients with AD and from healthy control subjects

To assess the effect of treatment with fixation and permeabilization on staining of surface CD4 and CD8 molecules, we examined frequencies of CD4⁺ cells and CD8⁺ cells in treated and untreated samples. Isolated PBMCs were stained with anti-cytokine mAbs, followed by staining of surface CD4 and CD8 antigens with or without fixation in 2% paraformaldehyde and with or without permeabilization in 0.1% saponin. There was no difference in the frequency of CD4⁺ or CD8⁺ cells between treated samples and untreated samples. Therefore we concluded that paraformaldehyde fixation and saponin permeabilization do not affect the detection of surface CD4 and CD8 antigens in our method.

The frequency of CD4⁺ cells from patients with AD ($44.6\% \pm 8.3\%$) was significantly higher ($p < 0.01$) than that from healthy control subjects ($37.9\% \pm 6.8\%$), whereas the frequencies of CD8⁺ cells from patients with AD and healthy control subjects ($22.7\% \pm 6.9\%$ and $23.5\% \pm 7.7\%$, respectively) did not show significant difference. However, white blood cell counts of patients with AD were higher than those of healthy control subjects with the result that absolute numbers of CD4⁺ and CD8⁺ cells in patients with AD were higher than those from healthy control subjects (data not shown).

Specificity of intracellular cytokine staining

PBMCs from two patients with AD were used for examining the specificity of intracellular cytokine staining. PBMCs from each patient were stimulated with immobilized anti-CD3 mAb in the presence of monensin for 6 hours. The harvested cells were stained with anti-IL-2 or anti-IL-4 mAb in the presence or absence of an excess of the corresponding recombinant cytokine and were analyzed by flow cytometry. Fig. 1 shows a one-parameter histogram for the frequency of cytokine-producing CD4⁺ cells. Anti-IL-2 mAb (Fig. 1, A) or anti-IL-4 mAb (Fig. 1, B) staining showed a distinct proportion of the CD4⁺ cells expressing each cytokine. Also, it showed that staining of the CD4⁺ cells with anti-cytokine mAbs was completely blocked in the presence of an excess amount of each corresponding recombinant cytokine. Similar results were obtained with CD8⁺ cells (data not shown). These data verified the specificity of this staining procedure.

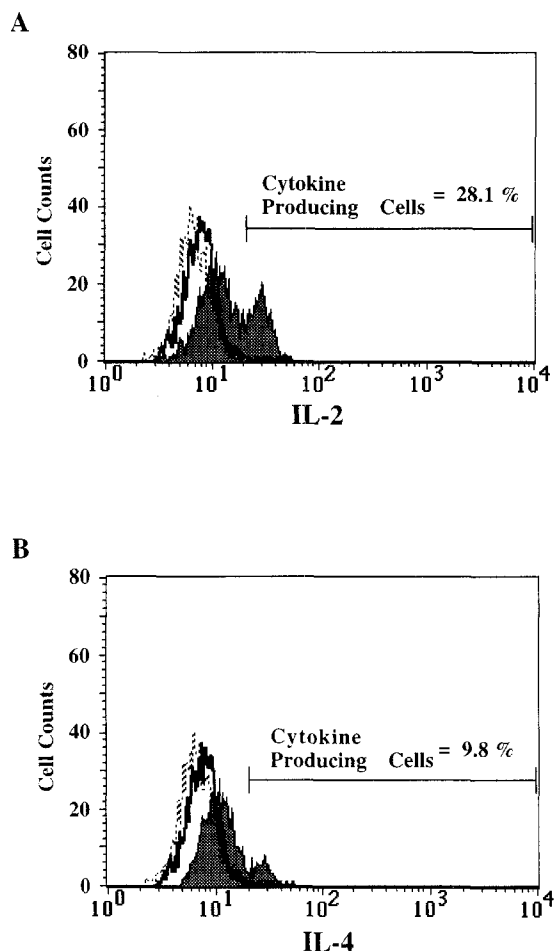


FIG. 1. PBMCs from a patient with AD were stimulated for 6 hours with immobilized anti-CD3 mAb in the presence of monensin. Cells were stained for IL-2 (A) or IL-4 (B), and CD4. An analysis gate was set on lymphocytes, by forward- and side-scatter properties, and CD4⁺ cells. The histograms illustrate intracellular staining obtained in the presence (solid line) or absence (shaded) of excess amounts of specific recombinant cytokines. Intensity of fluorescence blocked by excess amounts of recombinant cytokines was overlapped with intensity of fluorescence of the negative control (dotted line).

Kinetics of cytokine-producing cells in patients with AD after stimulation with anti-CD3 mAb

PBMCs from nine healthy control subjects and seven patients with AD (one patient with severe AD, two with moderate AD, and four with mild AD) were examined for kinetics of frequency of IL-2- or IL-4-producing cells after stimulation with immobilized anti-CD3 mAb. For the kinetics of IFN- γ -producing cells, four healthy control subjects and six patients with AD (two with severe AD, three with moderate AD, and one with mild AD) were examined. PBMCs from each patient or control subject were cultured with immobilized anti-CD3 mAb for 6 or 24 hours. Monensin (1 μ mol/L) was added to the culture for the last 6 hours. The frequency of cytokine-producing cells was analyzed immediately

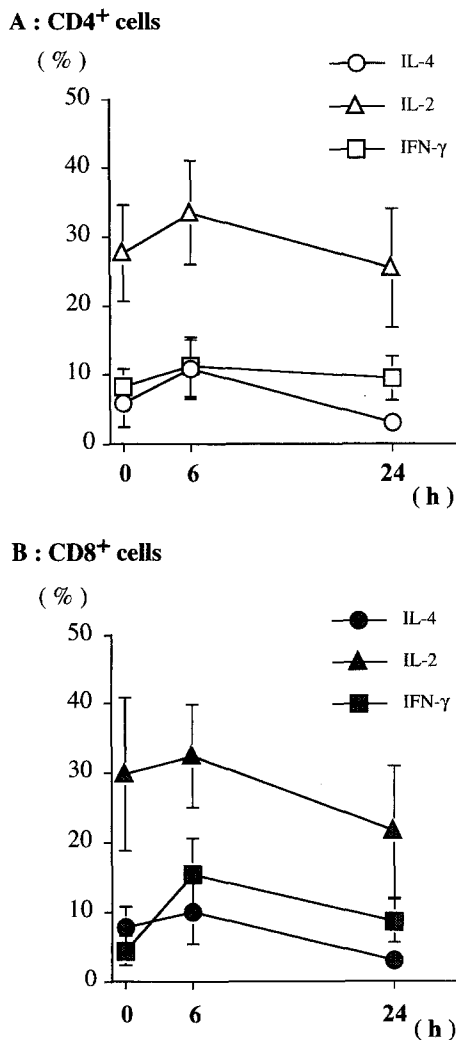


FIG. 2. PBMCs from patients with AD were stimulated for 6 or 24 hours with immobilized anti-CD3 mAb, and monensin was added for the last 6 hours. Cells were stained for IL-4, IL-2, or IFN- γ . Frequencies of cells producing each cytokine were examined at 0 hours (without stimulation) and after 6 and 24 hours of stimulation with anti-CD3 mAb. Frequency of cytokine-producing cells is shown as the mean percentage $\pm$ SD of the CD4⁺ (A) or the CD8⁺ cell population (B).

after isolation (0 hour) and 6 and 24 hours after stimulation.

The frequencies of IL-4-producing CD4⁺ cells from patients with AD were 5.7%, 10.7%, and 3.0% at 0, 6, and 24 hours after stimulation, respectively (Fig. 2, A). The frequencies of IL-2-producing CD4⁺ cells from patients with AD were 27.6%, 33.5%, and 25.4%; and the frequencies of IFN- γ -producing CD4⁺ cells were 8.2%, 11.1%, and 9.4%, respectively (Fig. 2, A). The frequencies of CD4⁺ cells producing each cytokine showed a peak at 6 hours of culture. In CD8⁺ cells of patients with AD, the frequencies of IL-4-producing cells were 7.7%, 10.0%, and 2.9% at 0, 6, and 24 hours after stimulation, respectively (Fig. 2, B). The frequencies of IL-2-producing CD8⁺ cells from patients with

AD were 29.8%, 32.4%, and 21.5%; and those of IFN- γ -producing CD8⁺ cells were 4.4%, 15.3%, and 8.6%, respectively (Fig. 2, B). Thus the frequency of CD8⁺ cells producing each cytokine also reached a peak at 6 hours after stimulation. The frequency of cells producing each type of cytokine within the CD4⁺ cells and CD8⁺ cells from healthy control subjects also reached a peak at 6 hours of culture (data not shown). Therefore we examined the frequency of cytokine-producing cells at 0 and 6 hours of culture in the following experiments.

Frequency of IL-4-, IFN- γ -, or IL-2-producing CD4⁺ cells in patients with AD

We examined the frequency of IL-4-, IFN- γ -, or IL-2-producing cells in CD4⁺ cells. The frequency of IL-4-producing CD4⁺ cells from patients with AD at the time of isolation (0 hour) was significantly higher than that from healthy control subjects (8.6% vs 3.1%, $p < 0.001$) (Fig. 3, A). After 6 hours of stimulation with anti-CD3 mAb in the presence of monensin, the frequency of IL-4-producing CD4⁺ cells from patients with AD increased to 14.8%, whereas the frequency of IL-4-producing cells from healthy control subjects showed no significant increase compared with the 0-hour frequency (Fig. 3, B). In contrast, the frequency of IFN- γ -producing CD4⁺ cells from patients with AD after 6 hours of stimulation was significantly lower than that from healthy control subjects (10.4% vs 21.3%, $p < 0.01$), whereas, the frequency of IFN- γ -producing CD4⁺ cells from patients with AD at 0 hour was comparable to that from healthy control subjects (Fig. 3, E and F). However, the frequencies of IL-2-producing CD4⁺ cells from patients with AD at 0 hours and 6 hours after stimulation were comparable to those from healthy control subjects (Fig. 3, C and D).

Frequency of IL-4-, IFN- γ -, or IL-2-producing CD8⁺ cells in patients with AD

The frequency of IL-4-producing CD8⁺ cells from patients with AD at 0 hours was significantly higher than that from healthy control subjects (11.0% vs 3.0%, $p < 0.001$) (Fig. 4, A). After 6 hours of stimulation with immobilized anti-CD3 mAb, the frequency of IL-4-producing CD8⁺ cells from patients with AD increased to 19.8%, whereas the frequency of IL-4-producing cells from healthy control subjects did not show a significant increase compared with the frequency at 0 hours (Fig. 4, B). In contrast, the frequency of IFN- γ -producing CD8⁺ cells from patients with AD at 0 hours was significantly lower than that from healthy control subjects (6.2% vs 10.4%, $p < 0.05$) (Fig. 4, E). After 6 hours of culture, the frequency of IFN- γ -producing CD8⁺ cells from patients with AD was also significantly lower than that from healthy control subjects (14.8% vs 24.2%, $p < 0.01$) (Fig. 4, F). However, there was no significant difference in the frequencies of IL-2-producing CD8⁺ cells between patients with AD and healthy control

CD4⁺ Cells

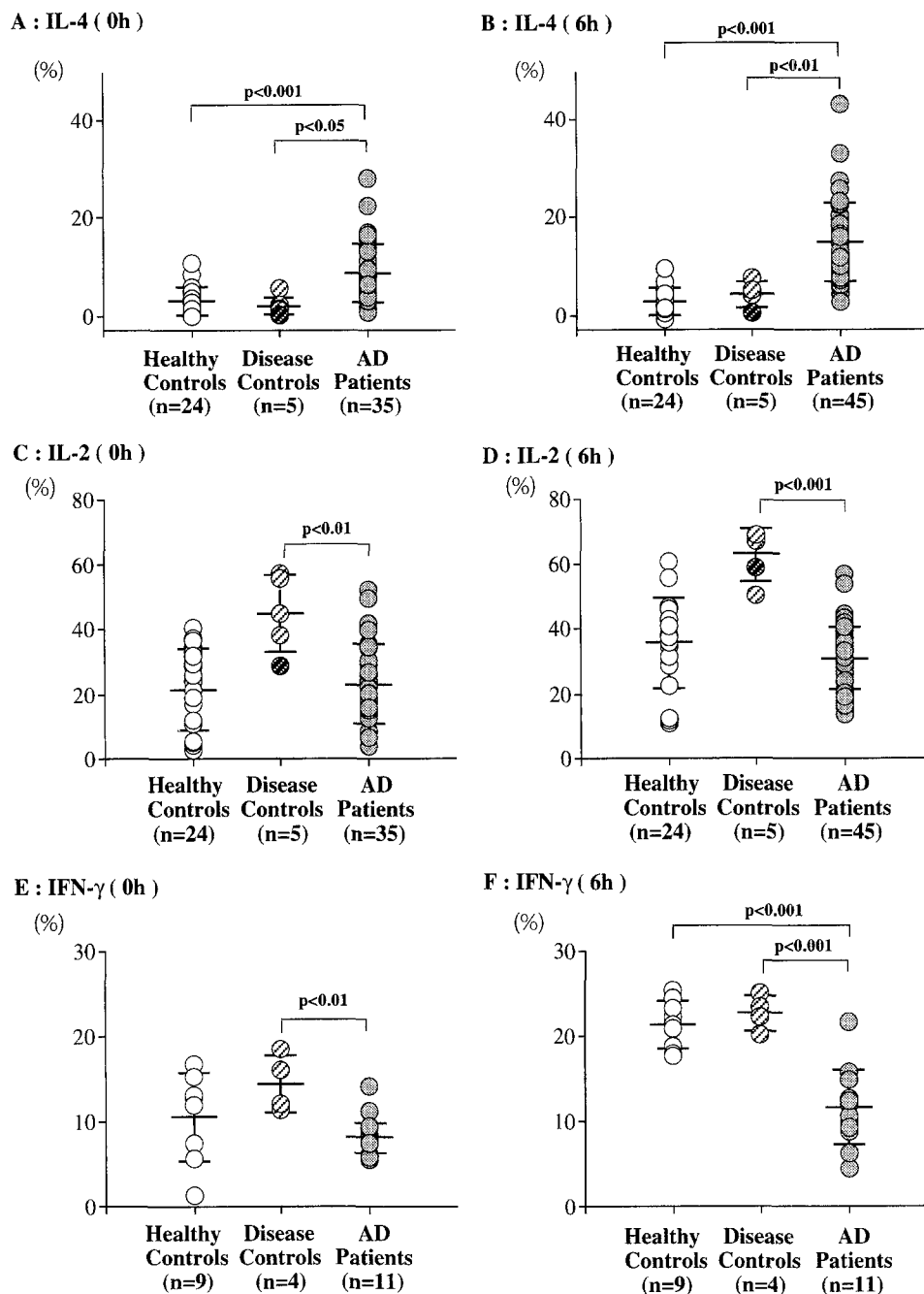


FIG. 3. PBMCs from patients with AD, disease controls (patients with chronic psoriasis [●] or contact dermatitis [⊙]), and healthy control subjects were stimulated for 6 hours with immobilized anti-CD3 mAb in the presence of monensin. Cells were stained for IL-4 (A and B), IL-2 (C and D), or IFN-γ (E and F). Frequencies of cells producing each cytokine were examined at 0 hours (without stimulation) (A, C, and E) and after 6 hours of stimulation with anti-CD3 mAb (B, D, and F). Frequency of cytokine-producing cells is shown as a percentage of the CD4⁺ cell population. Lines (±) show mean ± SD.

subjects at 0 and 6 hours after stimulation (Fig. 4, C and D).

Also, we examined the frequency of cytokine-producing cells from patients with chronic psoriasis

and a patient with contact dermatitis with mercury for the disease controls. The frequencies of IL-4-producing CD4⁺ or CD8⁺ cells from the disease controls were lower than those from patients with AD,

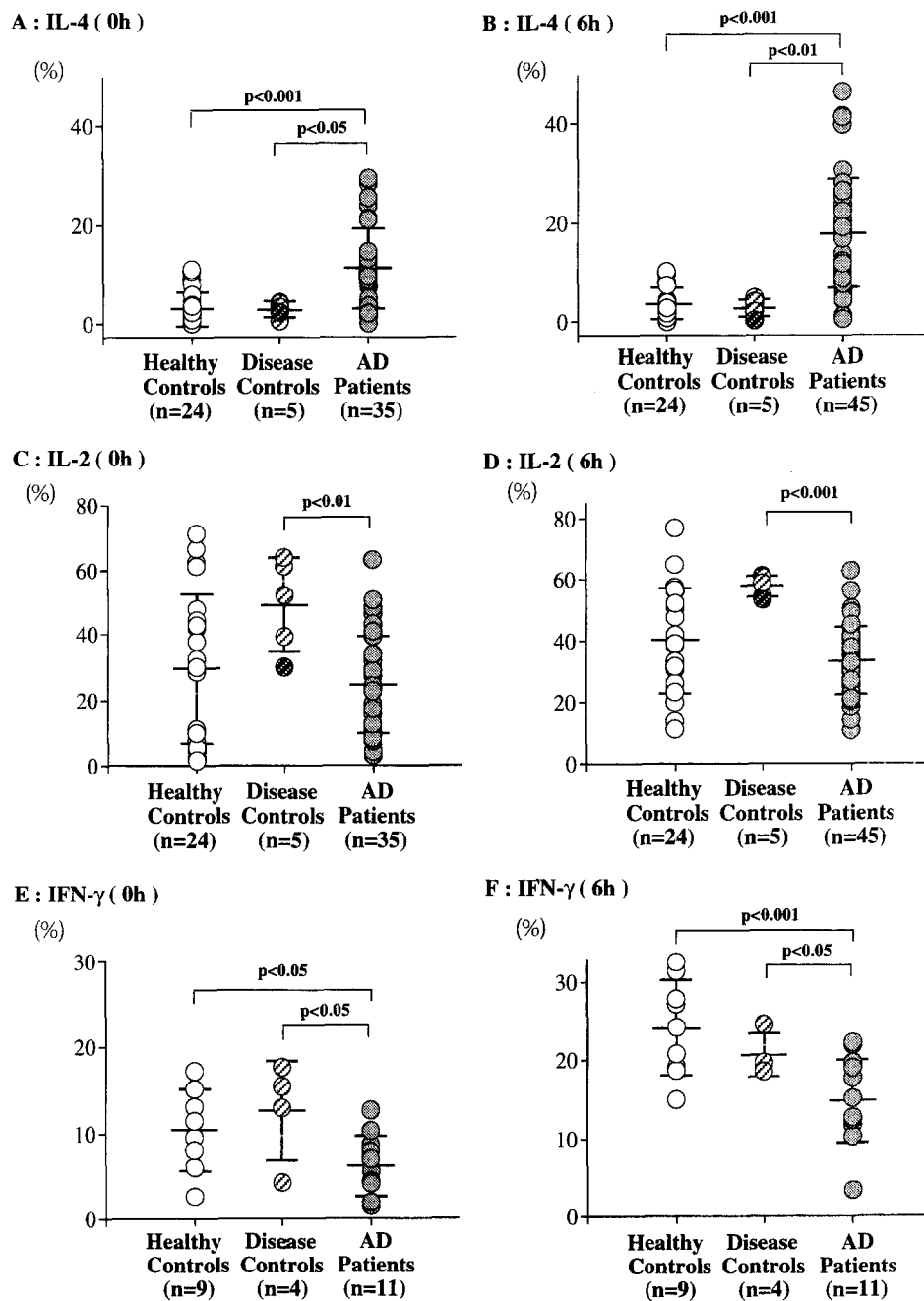
CD8⁺ Cells

FIG. 4. PBMCs from patients with AD, disease controls (patients with chronic psoriasis [●]) or contact dermatitis (◐), and healthy control subjects were stimulated for 6 hours with immobilized anti-CD3 mAb in the presence of monensin. Cells were stained for IL-4 (A and B), IL-2 (C and D), or IFN- γ (E and F). Frequencies of cells producing each cytokine were examined at 0 hours (without stimulation) (A, C, and E) and after 6 hours of stimulation with anti-CD3 mAb (B, D, and F). Frequency of cytokine-producing cells is shown as a percentage of the CD8⁺ cell population. Lines (±) show mean $\pm$ SD.

whereas the frequencies of IL-2- and IFN- γ -producing CD4⁺ or CD8⁺ cells from the disease controls were higher than those from patients with AD (Figs. 3 and 4).

Relationship between frequency of cells producing each cytokine and severity of AD

To study the relationship between the severity of AD and the frequency of cells producing each cytokine,

patients with AD were categorized into three groups by their symptoms according to the criteria of Rajka and Langeland.²⁹ For analysis of IL-2-producing cells and IL-4-producing cells, PBMCs were obtained from 11, 23, and 11 patients with severe, moderate, or mild AD, respectively. For analysis of IFN- γ -producing cells, PBMCs were obtained from three, six, and four patients with severe, moderate, or mild AD, respectively. PBMCs were cultured with immobilized anti-CD3 mAb for 6 hours, and cells were stained with anti-IL-4, anti-IFN- γ , or anti-IL-2 mAb in combination with anti-CD4 mAb and anti-CD8 mAb. The frequency of IL-4-producing CD4⁺ cells from the groups with severe ($19.2\% \pm 9.2\%$) or moderate ($15.0\% \pm 7.7\%$) disease was significantly higher ($p < 0.01$) than that from the group with mild AD ($9.4\% \pm 4.4\%$) (Fig. 5, A). As shown in Fig. 5, B, the frequency of IL-4-producing CD8⁺ cells from the severe group ($24.9\% \pm 11.5\%$) was significantly higher than that from the other groups (moderate AD, $15.9\% \pm 10.3\%$, $p < 0.05$; mild AD, $9.6\% \pm 6.4\%$, $p < 0.01$). Also, the frequency of IL-4-producing cells from the group with moderate AD was significantly higher ($p < 0.05$) than that from the mild AD group. In addition, the frequency of IL-4-producing cells in CD4⁺ cells ($3.2\% \pm 2.3\%$) and CD8⁺ cells ($3.7\% \pm 3.2\%$) from healthy control subjects was significantly lower ($p < 0.001$) than that from any of the groups of patients with AD (Fig. 5, A and B). As for the frequency of IFN- γ -producing CD4⁺ and CD8⁺ cells, there were no differences among the three groups. However, the frequencies of IFN- γ -producing CD4⁺ cells and CD8⁺ cells from patients with AD in any of the severity groups were significantly lower ($p < 0.01$) than those from healthy control subjects (Fig. 5, E and F). The frequencies of IL-2-producing cells among either CD4⁺ cells or CD8⁺ cells from all of the groups of patients with AD were comparable to the frequencies from healthy control subjects (Fig. 5, C and D).

DISCUSSION

In this article, we have demonstrated that the frequency of IL-4-producing CD4⁺ cells in PBMCs from patients with AD was significantly higher than that from healthy control subjects. In contrast, the frequency of IFN- γ -producing CD4⁺ cells from patients with AD was significantly lower than that from healthy control subjects when PBMCs were stimulated with immobilized anti-CD3 mAb for 6 hours. Also, among CD8⁺ cells, the frequency of IL-4-producing cells from patients with AD was significantly higher than that from healthy control subjects, whereas the frequency of IFN- γ -producing cells from patients with AD was significantly lower than that from healthy control subjects. The frequency of IL-4-producing cells within either CD4⁺ cells or CD8⁺ cells was well correlated with the severity of AD. However, the frequency of IL-2-producing cells within either CD4⁺ cells or CD8⁺ cells showed no significant difference between patients with AD and healthy control subjects. Also, our data showed that the

frequency of IL-4-producing cells in freshly isolated PBMCs from patients with AD was higher than that from healthy control subjects.

AD is characterized by enhanced IgE production.¹⁶⁻¹⁸ We confirmed that the patients with AD used in this analysis showed a higher level of IgE synthesis and that this was well correlated with severity of AD (data not shown). T_{H2} cytokines, especially IL-4, have been reported to be involved in the differentiation of B cells for IgE formation, and the effect of T_{H2} cytokines has also been shown to be counteracted by T_{H1} cytokines, especially IFN- γ .³⁰⁻³³ Therefore enhanced IgE synthesis in patients with AD may be caused by upregulation of T_{H2} cytokine-producing cells, especially IL-4-producing cells, and downregulation of T_{H1} cytokine-producing cells, especially IFN- γ -producing cells. After in vitro stimulation of PBMCs from patients with AD with certain stimuli, increased IL-4 synthesis and decreased IFN- γ synthesis have been observed when compared with PBMCs from healthy control subjects by means of ELISA.²²⁻²⁵ Furthermore, Hamid et al.³⁴ reported that skin lesions of patients with AD had increased numbers of cells expressing IL-4 messenger RNA but not IFN- γ mRNA. In our experiments the frequency of IL-4-producing CD4⁺ cells in PBMCs from patients with AD was significantly higher than that from healthy control subjects (Fig. 3, A and B). Rousset et al.²² and Tang et al.²⁴ have also demonstrated that IL-2 production by PBMCs or T-cell clones from patients with AD is comparable to that from healthy control subjects. Thus increased IL-4 synthesis and decreased IFN- γ synthesis results at least in part from expansion of IL-4-producing CD4⁺ cells and a decrease in IFN- γ -producing CD4⁺ cells in patients with AD. However, Jung et al.³⁵ have observed no increase in the frequency of IL-4-producing cells, whereas they have observed decreased frequency of IL-2- and IFN- γ -producing cells in patients with AD after stimulation with phorbol ester and ionomycin. This discrepancy between our findings and their findings may be due to a difference in the method, including mAbs used for staining intracellular cytokines or, alternatively, a difference in the severity of AD in the patients studied. The method for direct detection of intracellular cytokines has been developed since the method was reported for the first time by Anderson et al.³⁶ In some cases the intensity of fluorescence for IL-4 may be very low and difficult to detect. Therefore we used an indirect immunologic staining method that is more sensitive than the direct immunostaining method. Our data clearly showed that numbers of IL-4-producing CD4⁺ cells were greatly expanded in patients with AD.

Our data showed that numbers of IL-4-producing cells were increased not only among CD4⁺ cells but also among CD8⁺ cells in AD (Fig. 4, A and B). Fong et al.⁵ reported that CD8⁺ cells show a stronger bias toward differentiation into T_{H1}-like cytokine-producing cells compared with CD4⁺ cells. Naive CD4⁺ T cells could differentiate into IL-4-producing cells when stimulated

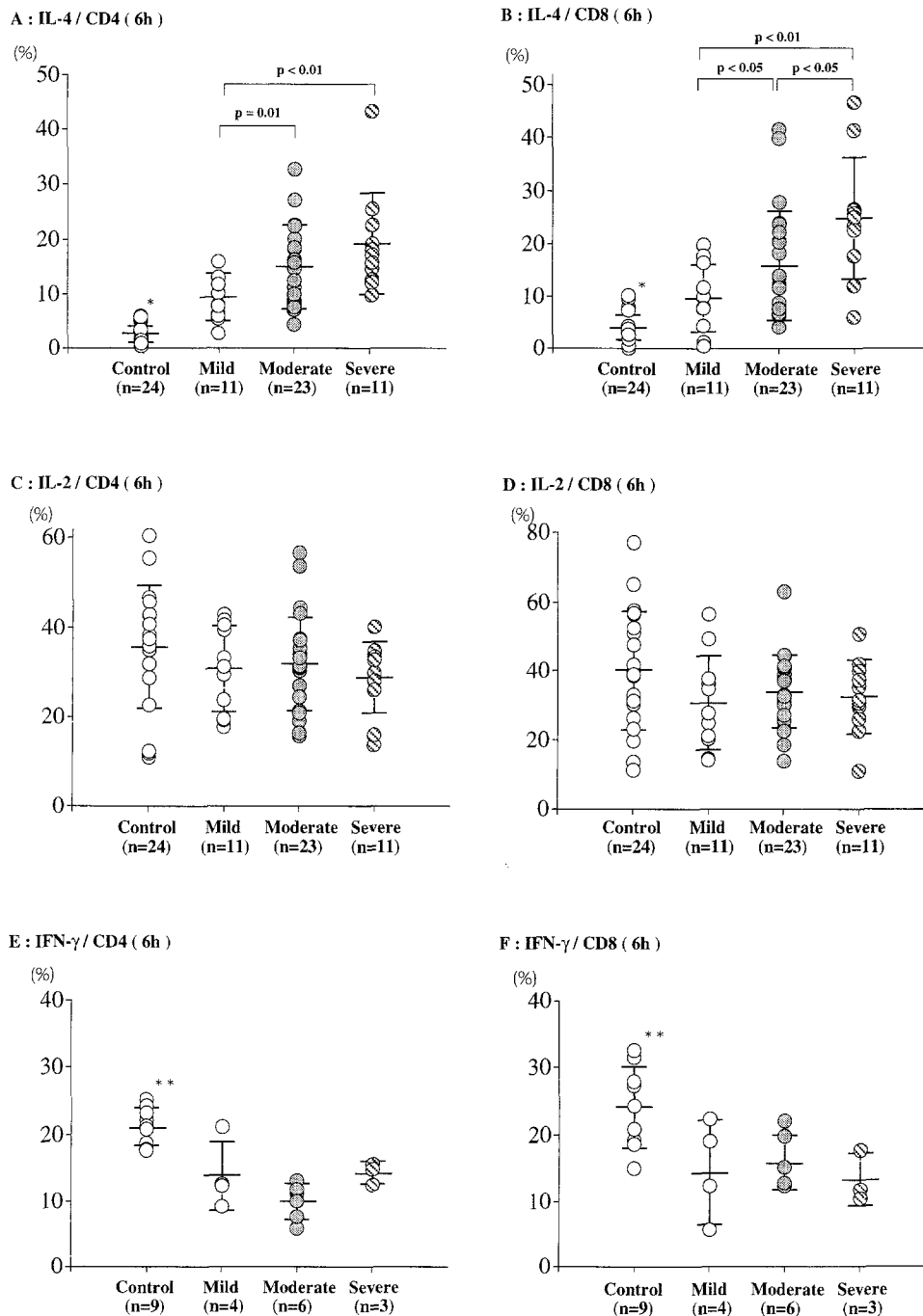


FIG. 5. PBMCs from patients with AD or healthy control subjects were stimulated for 6 hours with immobilized anti-CD3 mAb in the presence of monensin. Cells were stained for IL-4 (**A** and **B**), IL-2 (**C** and **D**), or IFN- γ (**E** and **F**). Frequency of cytokine-producing cells is shown as a percentage of the CD4⁺ cell (**A**, **C**, and **E**) or CD8⁺ cell (**B**, **D**, and **F**) population. Patients with AD were divided into three groups according to disease severity. Lines ($\pm$) show mean $\pm$ SD. *Frequency of IL-4-producing CD4⁺ and CD8⁺ cells from healthy control subjects was significantly lower ($p < 0.001$) than that from any group of patients with AD. **Frequency of IFN- γ -producing CD4⁺ and CD8⁺ cells from healthy control subjects was significantly higher ($p < 0.01$) than that from any group of patients with AD.

with IL-2 and mitogen in vitro,¹² whereas CD8⁺ T cells require exogenous IL-4, anti-IFN- γ antibody, or both for the differentiation into IL-4 producing cells.³⁷ The characteristics of cytokine production by CD8⁺ T cells also

reflect the status of diseases. Cytotoxic type 1 cytokine-secreting CD8⁺ T-cell clones (secreting IFN- γ but not IL-4) were derived from healed lesions of patients with tuberculoid leprosy, whereas T-cell clones that secreted

IFN- γ , IL-4, IL-5, and IL-10 were derived from patients with lepromatous leprosy who had high levels of antibody.⁹ Salgame et al.⁹ reported that the proliferation of *M. leprae*-specific CD4⁺ T-cell (T_{H1}) clones was inhibited by IL-4 secreted by CD8⁺ T cell clones. CD8⁺ T-cell clones secreting a more typical type 2-cytokine pattern (IL-4 and IL-5 but not IFN- γ) were derived from phytohemagglutinin-stimulated peripheral blood lymphocytes and skin biopsy specimens from HIV-infected individuals with a syndrome similar to Job's syndrome but not from normal control subjects.¹⁰ Also, IL-4 secreted by CD8⁺ T cells helps T_{H2} cell activity and humoral immune reactivity in experimental mouse models.³⁷ Thus our observations suggest that type 2 cytokine-producing CD8⁺ T cells in patients with AD inhibit T_{H1} cell activity and expand T_{H2} cells to produce type 2 cytokines. The increase in numbers of type 2 cytokine-producing CD4⁺ and CD8⁺ cells in PBMCs from patients with AD suggested that patients with AD have not only a skin problem but also a systemic allergic condition.

Finally, the frequency of type 2 cytokine-producing cells was well correlated with the severity of AD (Fig. 5), supporting the view that type 2 cytokine-producing cells play important roles in the maintenance of pathologic conditions in AD. Further, increased IL-4 synthesis and decreased IFN- γ synthesis in patients with AD could be caused by the expansion of type 2 cytokine-producing cells and a decrease in the number of type 1 cytokine-producing cells in both the CD4⁺ and CD8⁺ subsets. The precise regulator of polarization for type 2 cytokine secretion remains to be determined.

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