Increased Frequency of Interleukin 4 Producing CD4+ and CD8+ Cells in Peripheral Blood from Patients with Systemic Sclerosis

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ABSTRACT. ***Objective.*** To determine the relationship between the frequency of cytokine producing cells and systemic sclerosis (SSc), we examined the frequencies of interleukin 4 (IL-4) or IL-2 producing CD4+ or CD8+ T cells in peripheral blood mononuclear cells (PBMC) from patients with SSc.
Methods. PBMC from 23 SSc patients and 14 healthy controls were isolated from heparinized blood by density centrifugation. Purified PBMC were stimulated with immobilized anti-CD3 monoclonal antibody for 6 h in the presence of monensin. Cells were fixed, made permeable, and stained for intracellular cytokines in combination with staining for T cell surface markers, CD4, and CD8.
Results. The frequency of CD4+ T cells was negatively correlated with duration of SSc, whereas the frequency of CD8+ T cells was not correlated with duration. The frequencies of IL-4 producing (IL-4+) cells in CD4+ T cells and in CD8+ T cells from patients with SSc were both significantly higher than those from controls. In contrast, the frequencies of IL-2 producing (IL-2+) cells in CD4+ T cells and in CD8+ T cells from patients with SSc were both significantly lower than that from controls. Further, the ratios of IL-4+/IL-4+ + IL-2+ in CD4+ T cells and in CD8+ T cells were both negatively correlated with disease duration of SSc.
Conclusion. These results suggest that type 2 cytokine producing T cells, not only CD4+ T cells but also CD8+ T cells, have important roles in the pathogenesis of SSc, especially in the early phase of SSc. (J Rheumatol 2001;28:1252–8)

Key Indexing Terms:
SYSTEMIC SCLEROSIS   T CELL SUBSETS   INTERLEUKIN 2
INTERLEUKIN 4   FLOW CYTOMETRY

Systemic sclerosis (SSc) is a connective tissue disorder characterized by vascular abnormalities, cutaneous and visceral fibrosis, and a variety of serum antinuclear antibodies (ANA). The pathogenesis of SSc is still unclear, but a dysfunction of the immune system may be involved. Its most characteristic aspects are cutaneous sclerosis caused by increased synthesis of collagen, glycosaminoglycans (GAG), and other connective tissue substances by dermal fibroblasts[1]. Recent studies have indicated that the production of collagen and GAG by cultured fibroblasts is enhanced by several cytokines such as transforming growth factor-ß[2], insulin-like growth factor[3], endothelin 1[4], interleukin 4 (IL-4)[5], and IL-6[6]. Several investigators have

suggested IL-4 as one of the factors in the initiation of the fibrotic process[5,7]. Potential sources of IL-4 are T cells and mast cells.

Murine CD4+ T helper (Th) cells are composed of distinct functional subsets distinguished by their cytokine production[8,9]. Th1 cells secrete IL-2, interferon-γ (IFN-γ), and lymphotoxin, and Th2 cells produce IL-4 and IL-5. Recent studies have shown that cytokine production is not limited to CD4+ Th cells. Murine and human CD8+ T cell clones are also divided into 2 subtypes according to the profiles of cytokine production[10-12]. Type 1 CD8+ T cells produce IL-2 and IFN-γ, and type 2 CD8+ T cells produce IL-4, IL-5, and IL-10. Cytokine producing CD8+ T cells may also play a role in the regulation of balance of Th1 and Th2 cells[13]. It may be considered that the immunological abnormalities are caused by dysregulation of cytokine production not only by CD4+ T cells but also by CD8+ T cells.

Analysis of cytokine production has been approached by measuring protein or mRNA levels of each cytokine. Recently, flow cytometric analysis of cytokine producing cells has been developed[14,15]. We applied this method to detect intracellular cytokines of individual cells and determined the percentage of IL-2 or IL-4 producing CD4+ or CD8+ T cells in the peripheral blood from patients with SSc.

MATERIALS AND METHODS

Patients. Peripheral blood was obtained from 23 female patients with SSc (58.0 ± 8.9 years of age, mean ± SD), who fulfilled the American College of Rheumatology criteria for the classification of SSc[16]. Only 3 of the 23 patients had taken prednisolone, D-penicillamine, or bucillamine, but they had been stopped 7 months or more before the blood samples were collected. Control peripheral blood was obtained from 14 age matched healthy female volunteers (52.2 ± 3.6 years of age). Informed consent was obtained from the patients and volunteers.

The onset of disease was defined after careful questioning, which was either the beginning of Raynaud's phenomenon (RP) in most cases or awareness of numbness, puffiness, or sclerosis of fingers without preceding RP in a few cases. Pulmonary fibrosis was defined as present by radiographic findings and pulmonary function test. Esophageal involvement was assessed by radionuclide esophageal scintigraphy and contrast studies of the esophagus.

Monoclonal antibodies. Anti-human IL-2 and anti-human IL-4 monoclonal antibodies (Mab) (mouse IgG1) were purchased from Genzyme (Cambridge, MA, USA). Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG1 antibody was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA). Phycoerythrin (PE) conjugated anti-CD4 Mab and peridinin chlorophyll (PerCP) conjugated anti-CD8 Mab were purchased from Becton Dickinson (San Jose, CA, USA). Anti-CD3 Mab was isolated from culture supernatants of OKT3 cell line (American Type Culture collection, Rockville, MD, USA) using protein G Sepharose (PharMingen, San Diego, CA, USA).

Cell cultures. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll (Histopaque®-1077; Sigma, St. Louis, MO, USA) density centrifugation and washed 3 times with RPMI 1640 (Gibco BRL, Grand Island, NY, USA). PBMC (4×10^6 cells/ml) were cultured in 24 well culture plates (Sumitomo Bakelite, Tokyo, Japan) with RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (Biological Industries, Haemek, Israel), 100 U/ml penicillin (Banyu Pharmaceutical, Tokyo, Japan), 100 μg/ml streptomycin (Meiji Seika, Tokyo, Japan), 15 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer (Wako Junyaku, Osaka, Japan), 2×10^{-5} M 2-mercaptoethanol (Sigma), and 2 mM L-glutamine (Wako Junyaku). Cells were cultured for 6 h at 37°C in 5% CO_2 and 85% humidified incubator. The cells were stimulated with immobilized anti-CD3 Mab in the presence or absence of monensin (1 μM) (Sigma). Previously, we reported that the frequency of cytokine producing cells from patients with atopic dermatitis, Behçet's disease, or healthy controls showed a peak after 6 h stimulation with immobilized anti-CD3 Mab[17,18]. In this study, we examined the frequency of cytokine producing cells after 6 h *in vitro* stimulation.

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 20 min on ice. After 2 further washes in PBS, cells were resuspended to a concentration of 1×10^6 in 100 μl PBS containing 0.1% saponin, 10% human AB blood type serum, and 1 μg/ml goat IgG (Chemicon, Temecula, CA, USA). After 10 min, cytokine specific Mab was added at a concentration of 1 μg/ml for 30 min on ice. The cells were washed twice in PBS containing 0.1% saponin (saponin buffer) and resuspended in 100 μl saponin buffer with 1 μg/ml goat IgG and 1.5 μg/ml FITC conjugated goat anti-mouse IgG1 for 30 min in the dark at room temperature. Cells were washed once in saponin buffer, followed by 2 further washes in PBS, and then resuspended in 100 μl of PBS containing 2 μg/ml mouse IgG (Chemicon) for 10 min on ice. PE conjugated anti-CD4 Mab and PerCP conjugated anti-CD8 Mab were added for 30 min on ice. Finally, the cells were washed twice in PBS and resuspended in about 500 μl of PBS. For the negative control, cells were stained with FITC conjugated 2nd antibody without anti-cytokine Mab, followed by staining with anti-CD4 Mab and anti-CD8 Mab.

As preliminary experiments, the specificity of the staining procedure was confirmed, since the binding of each Mab was blocked with an excess amount of recombinant cytokine (IL-2 or IL-4; PharMingen) as described[17].

Flow cytometry. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) 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 from each sample were acquired into list mode and the data were analyzed with CELLQuest software (Becton Dickinson). Gates were set on lymphocytes according to forward and side scatter properties. Results were expressed as the percentage of cytokine producing cells in CD4+ and CD8+ T cell population.

Statistical analysis. Statistical analysis was performed using unpaired Student's t test.

RESULTS

Clinical characteristics. The individual data of age, disease duration, initial symptom, types of ANA, lung fibrosis, esophageal dysfunction, and extent of skin sclerosis[19,20] are shown in Table 1. The chest radiographic findings and the results of pulmonary function test are shown in Table 2. The duration of the disease was from one to 41 years with a mean of 15 years. The initial symptom was Raynaud's phenomenon in 17, numbness of fingers in 2, puffy fingers in one, sclerotic fingers in one, and unknown in 2 patients (Table 1). No significant correlation was found between the duration of SSc and the patient's age. Four patients (cases 12, 13, 18, 20) showed both radiographic fibrosis and decreased vital capacity (VC < 80%). Four patients (cases 5, 6, 7, 16) showed radiological fibrosis on radiography, but normal VC (> 90%) and DLCO (> 70%) (Table 2). Thus these 8 patients were defined as pulmonary fibrosis (Table 1). Fifteen patients had esophageal dysfunction. The patients were classified into 12 limited[20] (Barnett type 1[19]: 10 cases, type 2: 2 cases) and 11 diffuse (Barnett type 2: 3 cases, type 3: 8 cases) SSc (Table 1).

Percentage of CD4+ and CD8+ cells. To assess the effect of fixation and permeabilization on the staining of surface CD4 and CD8 molecules, we examined the percentage of CD4+ T cells and CD8+ T cells in treated and untreated samples. Isolated PBMC were stained for surface CD4 and CD8 antigens with or without fixation in 2% paraformaldehyde (PFA) and permeabilization in 0.1% saponin. There was no difference in the percentage of CD4+ or CD8+ T cells between treated samples and untreated samples (data not shown). We concluded that PFA fixation and saponin permeabilization do not modify the detection of surface CD4 and CD8 antigens with our method.

The percentage of CD8+ T cells from patients with SSc (23.3 ± 10.6%) was significantly higher (p < 0.05) than that from healthy controls (16.6 ± 4.0%), whereas the percentages of CD4+ T cells from patients with SSc and controls did not show significant difference (37.7 ± 10.3% and 38.1 ± 9.8%, respectively). However, there was no significant difference in the ratio of CD4+ to CD8+ T cells between the patients with SSc and the controls (2.16 ± 1.83 vs 2.52 ± 0.94, p = 0.495).

Figure 1A shows a negative correlation (r = –0.564, p =

Table 1. Clinical and biological features of patients with SSc studied.

Patient	Age, yrs	Duration, yrs	Initial symptom	ANA[a]	Lung[b]	Esophagus[c]	Barnett[d]	Type[e]
1	44	10	RP[f]	ACA	–	+	1	L
2	55	16	Numbness	RNP	–	+	3	D
3	48	21	Unknown	ACA	–	+	1	L
4	53	5	Puffy finger	ACA	–	–	3	D
5	63	19	RP	Topo-I	+	–	1	L
6	63	12	RP	ACA	+	+	2	L
7	60	14	RP	RNP	+	+	1	L
8	54	2	Numbness	Topo-I	–	–	3	D
9	48	8	RP	ACA	–	–	3	D
10	35	8	RP	Topo-I	–	+	2	D
11	67	6	Sclerotic finger	ACA	–	+	1	L
12	63	1	RP	Topo-I	+	+	3	D
13	58	23	RP	Topo-I	+	–	2	D
14	64	7	RP	ACA	–	–	1	L
15	64	22	RP	RNP	–	+	1	L
16	44	9	RP	Topo-I	+	+	3	D
17	57	3	RP	Others	–	+	2	D
18	69	41	Unknown	Topo-I	+	+	3	D
19	55	35	RP	RNP	–	–	1	L
20	63	16	RP	Topo-I	+	+	3	D
21	67	35	RP	ACA	–	+	1	L
22	63	17	RP	ACA	–	+	2	L
23	66	13	RP	Others	–	–	1	L

[a]ANA: antinuclear antibodies; ACA: anticentromere antibody; RNP: antiribonucleoprotein antibody; Topo-I: antitopoisomerase I antibody; Others: ANA other than ACA, RNP, and Topo-I.
[b]Lung: pulmonary fibrosis; [c]Esophagus: esophageal dysfunction; +: present; –: absent, assessed according to criteria described in Materials and Methods.
[d]Barnett: Barnett's classification[19].
[e]Type L: limited cutaneous SSc; D: diffuse cutaneous SSc[20].
[f]RP: Raynaud's phenomenon.

0.005) between the percentage of CD4+ T cells and the duration of SSc. There was no correlation between the percentage of CD8+ T cells and the duration of SSc (Figure 1B). The percentage of CD4+ and CD8+ T cells was not correlated with the age of SSc patients, or of healthy controls. The percentage of CD4+ and CD8+ T cells did not show significant differences among the patients' groups according to the type of ANA, the extent of skin sclerosis (Barnett's 3 types and diffuse vs limited), and the presence or absence of pulmonary fibrosis or esophageal dysfunction.

Frequency of IL-4 or IL-2 producing CD4+ and CD8+ cells. To examine the frequency of cytokine producing cells, purified PBMC were stimulated with immobilized anti-CD3 Mab. A representative result of the flow cytometric analysis is shown in Figure 2. The frequency of IL-4 producing CD4+ T cells from the patients with SSc (5.6 ± 2.5%) was significantly higher ($p < 0.001$) than that from controls (2.7 ± 1.7%) (Figure 3A). The frequency of IL-4 producing CD8+ T cells from the patients with SSc (5.5 ± 3.1%) was also significantly higher ($p < 0.05$) compared to controls (3.6 ± 2.3%) (Figure 4A). In contrast, the frequency of IL-2 producing CD4+ T cells from SSc patients (14.7 ± 6.5%) was significantly lower ($p < 0.01$) compared to controls (25.3 ± 16.7%) (Figure 3B). The frequency of IL-2

producing CD8+ T cells from the SSc patients (10.5 ± 6.0%) was also significantly lower ($p < 0.001$) than from controls (27.3 ± 16.6%) (Figure 4B). The frequencies of cytokine producing cells showed no significant differences among the patient groups according to the type of ANA, the extent of skin sclerosis (Barnett's 3 types and diffuse vs limited), and the presence or absence of pulmonary fibrosis or esophageal dysfunction.

Relationship between frequencies of cytokine producing cells and duration of SSc. We calculated the ratio of IL-4 producing T cells in IL-4 producing T cells plus IL-2 producing T cells, and indicated as IL-4/IL-4 + IL-2. Figure 5A shows a significantly negative correlation (r = –0.412, p = 0.05) between IL-4/IL-4 + IL-2 in CD4+ T cells and the duration of SSc. IL-4/IL-4 + IL-2 in CD8+ T cells also had a significantly negative correlation (r = –0.526, p = 0.01) with the duration of SSc (Figure 5B). However, the percentage of IL-4/IL-4 + IL-2 in both of CD4+ T cells or CD8+ T cells did not show any significant correlation with the age of controls or patients (data not shown).

DISCUSSION
Although several investigators have assessed the ratio of CD4+/CD8+ cells in peripheral blood from patients with

Table 2. Chest radiographic findings and the results of pulmonary function test.

Patient	CT/X-ray	% VC	%[$FEV_{1.0}$%]	%DLCO
1	N/N	102	90.9	104
2	N/N	105	102	105
3	N/N	90.8	112	70.1
4	N/N	133	110	97.4
5	F/F	95.3	96.2	80.7
6	F/F	119	105	106
7	F/F	92.5	103	77.2
8	N/N	97.1	84.0	111
9	N/N	92.5	103	77.2
10	N/N	103	107	119
11	N/N	94.0	80.4	ND
12	F/F	74.5	116	79.3
13	F/F	70.0	106	73.3
14	N/N	133	83.5	97.4
15	N/N	82.9	60.8	85.7
16	F/F	92.8	99.0	74.5
17	N/N	114	104	111
18	F/F	53.9	98.4	55.0
19	N/N	107	81.5	78.9
20	F/F	43.2	132	124
21	N/N	87.6	89.3	121
22	N/N	84.7	101	71.7
23	N/N	ND	ND	ND

CT: computerized tomography.
N: no fibrotic changes.
F: fibrotic changes.
ND: not done.

SSc, there have been some discrepancies in the results. Keystone, *et al*[21] and Whiteside, *et al*[22] showed increased CD4+/CD8+ ratio, whereas Gupta, *et al*[23] reported decreased CD4+/CD8+ ratio in SSc patients compared to healthy controls. We found no significant differences in CD4+ T cells and in CD4+/CD8+ ratio between the SSc patients and controls; however, CD8+ T cells were significantly increased in SSc compared to controls. An increased number of CD8+ T cells in bronchoalveolar lavage fluids[24] and production of type 2 cytokines by CD8+ T cells in patients with SSc[25] have been reported. Recently clonally expanded CD8+ T cells have been shown to be associated with the clinical syndrome of primary biliary cirrhosis and limited scleroderma[26]. Keystone, *et al*[21] showed a higher ratio of CD4+/CD8+ in early disease compared to late disease. In our study the percentage of CD4+ showed a significantly negative correlation with disease duration, but that of CD8+ cells was not significantly related to duration (Figure 1). Taken together, these results suggest that CD4+

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Figure 2. Representative histogram of the flow cytometric analysis. PBMC were stimulated for 6 h with immobilized anti-CD3 Mab in the presence of monensin. Cells were stained with anti-human IL-4 Mab and FITC conjugated 2nd antibody, and then with PE conjugated anti-CD4 Mab. They were then subjected to flow cytometric analysis. IL-4+ CD4+ cells are shown in the upper right quadrant.
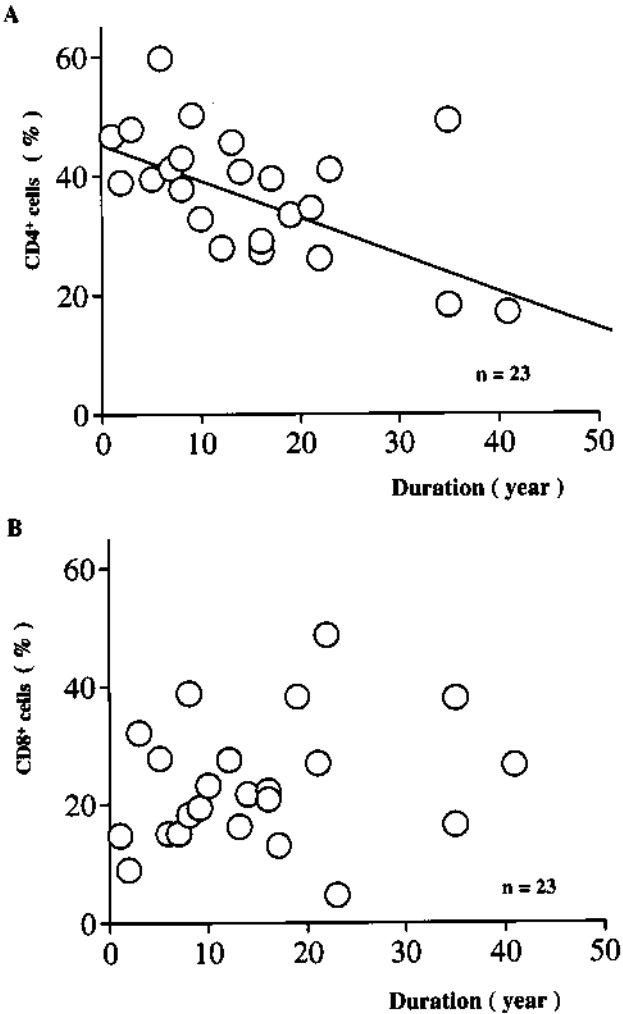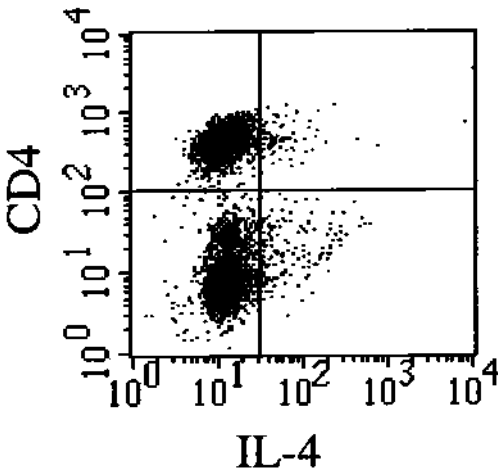

Figure 1. Correlation between the percentage of CD4+ cells or CD8+ cells in the peripheral blood and disease duration. A. The percentage of CD4+ cells has a negative correlation with disease duration (r = –0.564, p = 0.005). B. There was no significant correlation between the percentage of CD8+ cells and disease duration.

A

P < 0.001

IL-4 producing cells in CD4+ cells (%)

Controls
(n=14)

SSc Patients
(n=23)

B

P < 0.01

IL-2 producing cells in CD4+ cells (%)
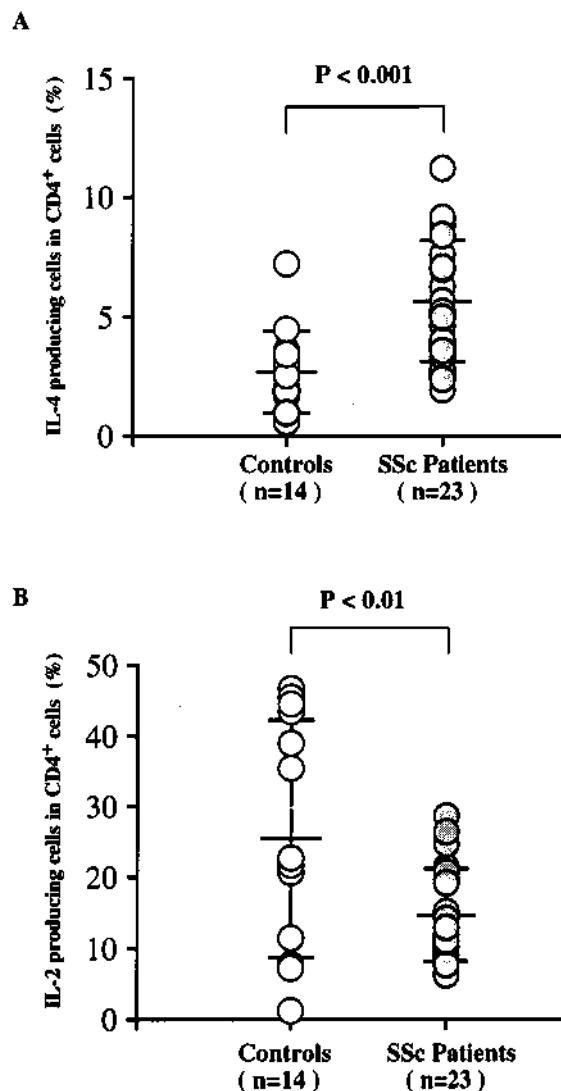
Controls
(n=14)

SSc Patients
(n=23)

Figure 3. Percentage of IL-4 or IL-2 producing CD4+ cells in SSc patients and controls. PBMC were stimulated for 6 h with immobilized anti-CD3 Mab in the presence of monensin. Cells were stained for IL-4 (A) or IL-2 (B). Percentage of cytokine producing cells is shown as that in the CD4+ cells. Bars indicate mean ± SD.

A

P < 0.05

IL-4 producing cells in CD8+ cells (%)

Controls
(n=14)

SSc Patients
(n=23)

B

P < 0.001

IL-2 producing cells in CD8+ cells (%)

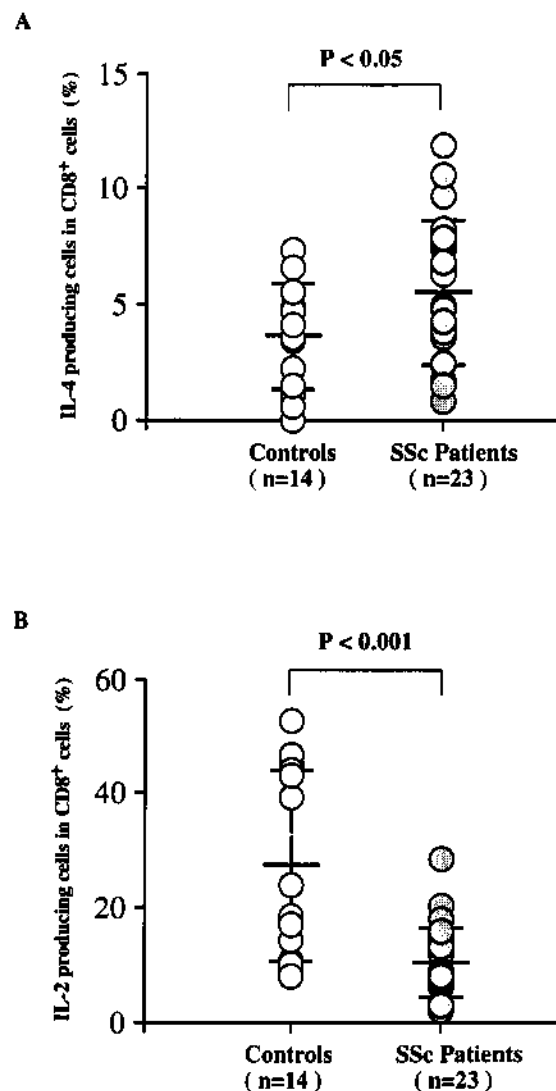Controls
(n=14)

SSc Patients
(n=23)

Figure 4. Percentage of IL-4 or IL-2 producing CD8+ cells in SSc patients and controls. PBMC were stimulated for 6 h with immobilized anti-CD3 Mab in the presence of monensin. Cells were stained for IL-4 (A) or IL-2 (B). Percentage of each cytokine producing cells is shown as that in the CD8+ cells. Bars indicate mean ± SD.

cells expand in the early phase of SSc and may contribute to the onset of SSc and that CD8+ cells may be the effector cells all through the course of SSc.

Elevated levels of serum IL-2, IL-4, and IL-6 detected by ELISA[27-29] and expression of IL-4 and IL-4 mRNA in lesional skin assessed by immunohistochemistry and *in situ* hybridization[5] have been reported in patients with SSc. IL-4 has been shown to induce the production of extracellular matrix components by fibroblasts[30,31]. The major source of IL-4 is supposed to be Th2 cells. However, several investigators have reported that human CD8+ cell clones secrete both type 1 and type 2 cytokines[12,32,33]. Salgame, *et al*[34] reported that the proliferation of *Mycobacterium leprae-*

specific Th1 clones was inhibited by IL-4 secreted from CD8+ T cell clones. These observations suggest that both CD4+ and CD8+ T cells may play important roles in SSc. However, it is still unclear whether such increased cytokine production is caused by increased numbers of cytokine producing cells or enhanced ability of cytokine production. Thus, we investigated the frequency of cytokine producing CD4+ T cells and CD8+ T cells in the peripheral blood at the single cell level by flow cytometry.

We demonstrated that the frequency of IL-4 producing CD4+ T cells in peripheral blood from patients with SSc was significantly higher than that from healthy controls. In contrast, the frequency of IL-2 producing CD4+ T cells in
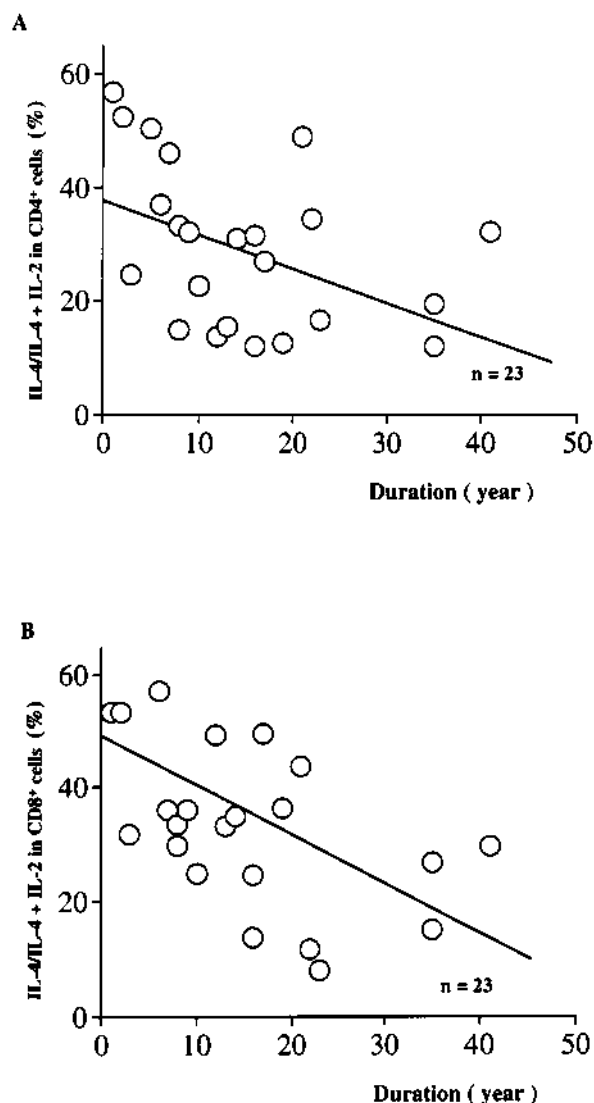
A

B

Figure 5. Correlation between the percentage of IL-4/IL-4 + IL-2 in CD4+ cells or CD8+ cells and disease duration. *X* axis shows disease duration. *Y* axis shows the percentage of IL-4 producing cells in IL-4 producing cells plus IL-2 producing cells. A. The percentage of IL-4/IL-4 + IL-2 in CD4+ cells (r = –0.412, p = 0.05). B. Percentage of IL-4/IL-4 + IL-2 in CD8+ cells (r = –0.526, p = 0.01).

peripheral blood from patients with SSc was significantly lower than from controls (Figure 3). As for CD8+ T cells, we also showed increased frequency of IL-4 producing cells and decreased frequency of IL-2 producing cells in peripheral blood from SSc patients compared to controls (Figure 4). These findings suggest that increased frequency of IL-4 producing (IL-4+) CD4+ T cells and CD8+ T cells may be one reason for increased type 2 cytokine production in patients with SSc.

Further, the ratio of IL-4+ cells/IL-4+ + IL-2+ cells in CD4+ or CD8+ T cells was negatively correlated with disease duration (Figure 5), but not with the age of patients.

These findings indicate that type 2 cytokine producing CD4+ and CD8+ T cells, especially IL-4 producing CD4+ T cells, are expanding in a relatively early stage of SSc and suggest that they might have an important role in the pathogenesis of SSc, especially for the induction of extracellular matrix production by fibroblasts. It may be necessary to study a more uniform population including patients in earlier stages, whose duration is less than 12 months, to verify our conclusion. Effective treatment for suppression of type 2 cytokine production during the early phase of SSc will be of great benefit to the management of the disease.

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