Vertebral Fracture and Bone Mineral Density in Women Receiving High Dose Glucocorticoids for Treatment of Autoimmune Diseases

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ABSTRACT. ***Objective.*** To evaluate the factors influencing the occurrence of vertebral fracture in patients receiving high dose glucocorticoids (GC).

Methods. A cross-sectional study was performed on women who had received at least 0.5 mg/kg of oral glucocorticoid for the treatment of autoimmune diseases for more than 1 month between 1998 and 2003. Logistic regression analysis and chi-square test were used to examine the effects of glucocorticoid dose and other factors on vertebral fractures. Receiver-operating characteristics curve (ROC) analysis was used to determine the bone mineral density (BMD) cutoff value for the risk of vertebral fracture.

Results. The study population comprised 160 women, including 35 with vertebral fractures. In ROC analysis, the BMD threshold of the risk of fracture for postmenopausal women (0.787 g/cm^2, T score −2.1) was lower than that for premenopausal women (0.843 g/cm^2, T score −1.7). Among patients with fractures, 7 of 16 premenopausal patients had normal BMD values (T score > −1), whereas only one of 19 postmenopausal patients showed a comparable level of BMD. Additionally, vertebral fracture was more frequent for patients with high total cholesterol values (> 280 mg/dl) than for those with normal total cholesterol values (< 220 mg/dl). Moreover, patients with high total cholesterol values had lower BMD values than those with normal total cholesterol values.

Conclusion. The fact that vertebral fracture frequently occurred in premenopausal patients with normal BMD and evidence that hyperlipidemia correlated with fracture suggest the pathology of vertebral fracture secondary to high dose glucocorticoid therapy is multifactorial and possibly involves lipid metabolism. (J Rheumatol 2005;32:863–9)

Key Indexing Terms:

OSTEOPOROSIS	VERTEBRAL FRACTURE	GLUCOCORTICOID
MENOPAUSE	BONE MINERAL DENSITY	HYPERLIPIDEMIA

Glucocorticoids are widely used for the treatment of a variety of autoimmune diseases. Even now, when various novel drugs for the treatment of these diseases are being introduced, glucocorticoids remain the main drugs of choice. However, it has been well established that the use of glucocorticoids can lead to rapid loss of bone mineral density

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(BMD) and to an increased risk of fracture[1]. Several epidemiologic studies have reported a doubling of the risk of hip fracture for users of glucocorticoids[2-4], while large-scale studies have demonstrated a rapid increase in fracture risk following the start of glucocorticoid therapy and a strong correlation of risk with daily glucocorticoid dose[4,5]. Other smaller studies have shown that the cumulative dose, rather than the daily dose, was the more reliable and accurate predictor of fracture[6,7]. When high dose glucocorticoids are used, the loss of bone such as vertebrae can be rapid and lead to vertebral compression fractures within a few months.

Glucocorticoids are also known to affect bone through various pathways, affecting mainly bone formation and, to a lesser extent, bone resorption[8,9]. Findings have been accumulating about the possible role of micro-architectural changes in glucocorticoid induced fracture, although fracture in glucocorticoid users may also occur simply as a result of bone loss. A recent hypothesis is that osteocyte apoptosis is an important factor in deterioration of bone quality and the concomitant rapid increase in the risk of fracture[10]. In addition, there is a report that glucocorticoid users with fracture had considerably higher BMD than patients with fracture due to primary osteoporosis[11]. These reports support the notion that a non-BMD-related mechanism may also be responsible for inducing fracture in users of glucocorticoids[12].

We conducted a multicenter, cross-sectional analysis, specifically investigating high dose glucocorticoid users treated for autoimmune diseases, to determine the BMD cutoff value for the risk of vertebral fracture, and to examine the correlation between glucocorticoid induced vertebral fracture or loss of BMD and multiple factors including menopause, glucocorticoid dose, and other glucocorticoid induced secondary complications.

MATERIALS AND METHODS

Study population of glucocorticoid users. Data on 160 Japanese women, aged 16–85 years and treated with glucocorticoids for autoimmune diseases, were collected from the rheumatology departments of 11 institutions that joined the Research Committee for Glucocorticoid-Induced Osteoporosis organized by the Japanese Ministry of Health, Labor and Welfare. This study was limited to patients who had been receiving oral glucocorticoid therapy (mean daily dose 0.5 mg/kg prednisone or equivalent) for at least 1 month between April 1998 and March 2003. The basic clinical data including risk factors and dose and duration of glucocorticoid therapy were collected retrospectively by treating physicians in reference to medical records from each institution, and the collected data were reviewed by the central committee for selecting eligible patients. As for treatment or prevention of osteoporosis, there were no restrictions for enrollment of patients based on protocols for the use of bisphosphonates, calcium, vitamin D, or other antiresorptive drugs. Diseases they were treated for included systemic lupus erythematosus (SLE; 79 cases), Sjögren's syndrome (15 cases), polymyositis (13 cases), mixed connective tissue disease (12 cases), adult onset Still's disease (8 cases), polymyalgia rheumatica (7 cases), dermatomyositis (6 cases), systemic sclerosis (5 cases), and others (15 cases). Patients with rheumatoid arthritis were excluded from this study.

BMD of the patients was assessed for the lumbar spine (L2–L4), femoral neck, and radial head by means of dual-energy x-ray absorptiometry (DEXA). Since the DEXA machines used for the measurement of BMD differed from hospital to hospital, the raw BMD values were converted to comparable values for the QDR-2000 (Hologic Inc., Waltham, MA, USA) as described[13]. High dose glucocorticoid therapy was defined as a mean daily dose > 0.5 mg/kg of prednisone or equivalent dose of other glucocorticoids for at least 1 month.

Vertebral fracture was confirmed radiologically by lateral radiographs of the thoracolumbar spine with the method established by Orimo, *et al*[14]; the presence of vertebral fracture was semiquantitatively confirmed if either the ratio of middle/anterior or middle/posterior height of a vertebral body was < 0.8, or the ratio of anterior/posterior height of a vertebral body was < 0.75. The judgment of fracture was double-checked by 2 examiners in each institution. If BMD was measured more than once in the same patient, the last BMD value was adopted for patients without vertebral fracture, and for patients with fracture, the BMD measured at the timepoint nearest the radiological confirmation of initial vertebral fracture was used.

The daily, cumulative, and maximum glucocorticoid doses, and the total duration (in days) of prior glucocorticoid therapy were also entered into the analysis. Clinical factors that may affect the occurrence of vertebral fracture, comprising age, body mass index (BMI), menopause, BMD (T scores), hypertension, total cholesterol, and HbA1c were evaluated. Diagnoses for hypertension and diabetes mellitus were determined according to American Heart Association[15] and American Diabetes Association[16] guidelines, respectively. Hyperlipidemia was diagnosed according to the criteria of the Japanese Atherosclerosis Society[17], in which total cholesterol level > 220 mg/dl is regarded as hyperlipidemia.

Statistical analysis. Logistic regression analysis was used to calculate the influence of various variables on vertebral fracture including age, BMI, menopause, BMD, and glucocorticoid related parameters. For determination of BMD cutoff values to identify women with vertebral fracture, sensitivity, specificity, and BMD cutoff values were calculated using receiver-operating characteristics curve (ROC) analysis. As for patients with vertebral fracture, the chi-square test was used to determine the difference in BMD between premenopausal and postmenopausal glucocorticoid users. P values < 0.05 were deemed to be statistically significant. The MedCalc statistical analysis software package (MedCalc Software, Mariakerke, Belgium) was used for statistical analyses.

RESULTS

Variables affecting vertebral fracture in high dose glucocorticoid users. For this study, 160 patients were assessed. The baseline information of enrolled patients is shown in Table 1. BMD values of this group negatively correlated with patients' age ($p < 0.001$, $r = -0.366$). A logistic regression analysis of patients with vertebral fracture (fracture group) and those without vertebral fracture (non-fracture group) is presented in Table 2. The respective mean BMD values of the fracture group (35 cases; 19 postmenopausal, 16 premenopausal) and the non-fracture group (125 cases) were 0.781 and 0.871 g/cm^2 ($p = 0.004$). There was a significant difference between the 2 groups in BMI and BMD, but no difference in age, ratio of menopause, and total glucocorticoid dose, as shown in Table 2. The logistic regression analyses including the other glucocorticoid related variables such as cumulative days of glucocorticoid use, mean glucocorticoid dose (daily), cumulative glucocorticoid dose, and maximal glucocorticoid dose showed no significant difference between the 2 groups (data not shown). The mean daily glucocorticoid dose for premenopausal women (age 34.9 ± 9.4 yrs) was 16.4 ± 16.5 mg/day and for postmenopausal

Table 1. Baseline characteristics of 160 patients in the study.

	Premenopausal	Postmenopausal	Total	p
Age, yrs, mean ± SD	34.9 ± 9.4	62.6 ± 9.9	47.9 ± 16.9	< 0.05
BMI, kg/m^2	21.7 ± 14.1	22.0 ± 3.5	21.9 ± 3.6	NS
BMD, g/cm^2	0.926 ± 0.149	0.767 ± 0.149	0.852 ± 0.168	< 0.05
Daily prednisolone dose*, mg/day	16.4 ± 16.5	10.7 ± 9.9	13.7 ± 14.1	< 0.05
Cumulative dose of prednisolone*, g	17.1 ± 31.3	8.2 ± 10.4	12.8 ± 24.0	NS
Duration of glucocorticoid treatment, days	1993.1 ± 2091.9	2069.9 ± 2317.4	2027.8 ± 2189.4	NS

* Adjusted to the dose equivalent to prednisolone. NS: not significant.

Table 2. Logistic regression analysis of treatment related variables and vertebral fracture in high dose user of glucocorticoid.

	Vertebral Fracture			
	Yes	No	Z	p
Age, yrs, mean ± SD	50.7 ± 3.2*	47.1 ± 1.4	0.5925	0.554
Menopause (%)	19/35 (54.3)	56/125 (44.8)	0.270	0.787
BMI	22.4 ± 0.8	21.8 ± 0.3	1.961	< 0.05
BMD, L2–4, g/cm^2	0.781 ± 0.033	0.871 ± 0.014	2.218	< 0.03
Total glucocorticoid dose*, g	24.3 ± 6.6	22.2 ± 4.4	0.789	0.430

* Adjusted to the dose equivalent to prednisolone.

women (age 62.6 ± 9.9 yrs) 10.7 ± 9.9 mg/day ($p < 0.05$). Compared to postmenopausal glucocorticoid users, premenopausal glucocorticoid users had significantly higher average BMD (L2–L4) in the lumbar spine, femoral neck, and radial head (data not shown).

For postmenopausal women, the mean BMD value of the fracture group was significantly lower than that of the non-fracture group ($p < 0.01$), as shown in Figure 1. In contrast, there was no significant difference in BMD values between the fracture group and non-fracture group among premenopausal women. Of special interest is that 7 of the 16 premenopausal patients (43.7%) in the fracture group showed normal values (T score > –1), whereas only one of the 19 postmenopausal patients (5.3%) did ($p < 0.01$). There was no statistically significant difference between the fracture group and non-fracture group for maximum glucocorti-

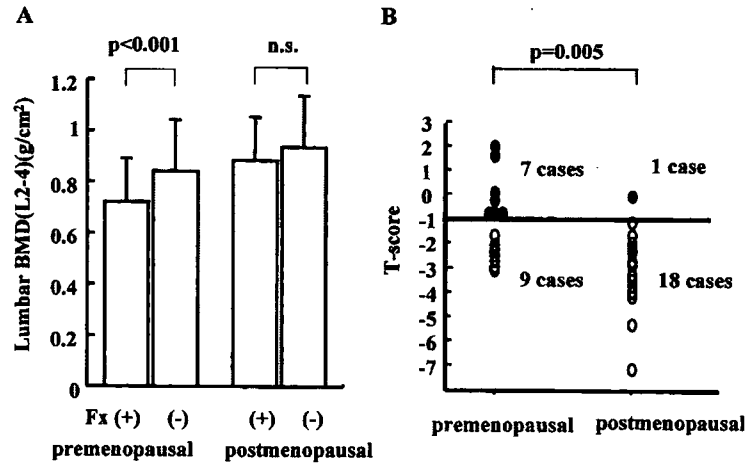

Figure 1. (A) Lumbar BMD from fracture (Fx) and non-fracture patient groups taking high dose glucocorticoids. There were significant differences in lumbar BMD between fracture and non-fracture groups in premenopausal women ($p < 0.001$), whereas no difference was detected between the 2 groups in postmenopausal women. ns: not significant. (B) T scores from premenopausal or postmenopausal women with vertebral fracture. Premenopausal glucocorticoid users frequently incurred vertebral fracture even when BMD was not reduced (T > –1) compared with postmenopausal women ($p = 0.005$). ●: fracture patients whose T scores were not reduced.

coid dose, mean daily glucocorticoid dose, disease background, and history of methylprednisolone pulse therapy in premenopausal women (data not shown).

BMD cutoff values for vertebral fracture in glucocorticoid users assessed by ROC analysis. ROC analysis was used to determine the BMD cutoff level for vertebral fracture in high dose glucocorticoid users. The cutoff values were defined as the values that proved to be effective for the sensitive and specific differentiation of subjects with and without vertebral fracture. As shown in Figure 2, the cutoff values for the risk of vertebral fracture for premenopausal, postmenopausal, and total patients were 0.843, 0.787, and 0.787 g/cm^2, respectively.

Hyperlipidemia correlates with BMD value and vertebral fracture. The influence of common glucocorticoid induced complications such as hyperlipidemia, diabetes mellitus, and hypertension on vertebral fracture were not entered into the logistic regression analysis, since those variables are not recognized as independent to glucocorticoid dose-related variables. Table 3 shows that hyperlipidemia has negative correlation with BMD, while HbA1c level did not correlate with BMD values. Nor did hypertension correlate with the level of BMD (data not shown). Then we compared patients with normal total cholesterol (< 220 mg/dl) value to those with above-normal values for further analysis. The peak value of total cholesterol after initiation of glucocorticoid therapy was used for the analysis in each patient. When we raised the comparative total cholesterol level to > 280 mg/dl, patients with high total cholesterol (> 280 mg/dl) value had lower BMD ($p = 0.016$) and higher risk of vertebral fracture (relative risk 3.1, $p = 0.032$) than those with normal total cholesterol level (Figure 3). These results suggest that hyperlipidemia following high dose glucocorticoid therapy may contribute to the risk for BMD reduction and vertebral fracture.

DISCUSSION

High dose glucocorticoid therapy is often the first choice for patients with autoimmune diseases, such as SLE, that frequently affect premenopausal women. Although the efficacy of bisphosphonate has recently been reported in high dose glucocorticoid users[18], there is only limited knowledge of the clinical risk factors for secondary osteoporosis occurring in high dose glucocorticoid users. This is the first extensive study focusing on the relationship of vertebral fracture and BMD in patients with high dose glucocorticoid therapy. We observed unique effects of high dose glucocorticoid therapy: First, the BMD cutoff value for the risk of vertebral fracture applicable to premenopausal glucocorticoid users was higher than that applicable to postmenopausal glucocorticoid users. Second, premenopausal glucocorticoid users, even with normal BMD values, were found to frequently incur vertebral fracture. Third, hyperlipidemia significantly correlated with vertebral fracture and low BMD.

ROC analysis showed that the BMD cutoff value for the risk of vertebral fracture for premenopausal women was 0.843 (T score = –1.7) and for postmenopausal women 0.787 (T score = –2.1). These cutoff values lie between 70% (T score = –2.6) and 80% (T score = –1.7) of the young adult

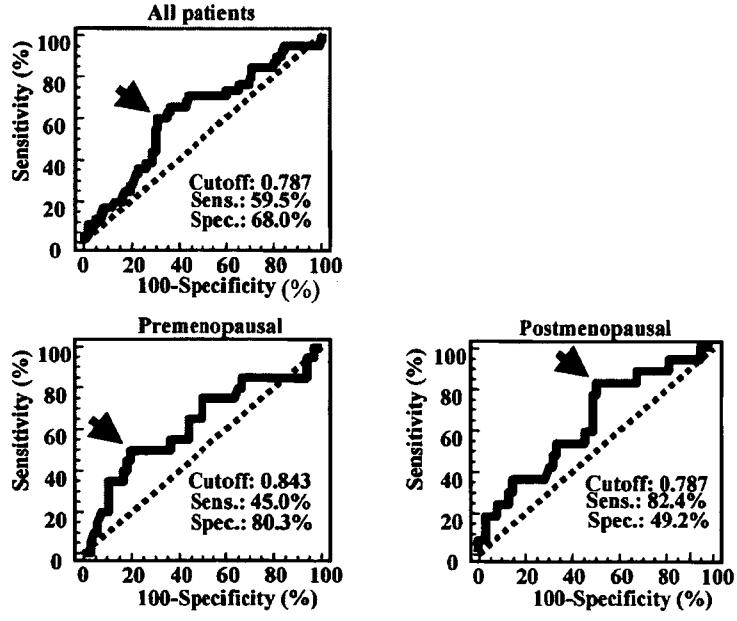

Figure 2. ROC analysis of lumbar BMD values for all patients, premenopausal and postmenopausal patients with vertebral fracture treated with high dose glucocorticoid. Arrows indicate cutoff points. Sens: sensitivity; Spec: specificity.

Table 3. The relationship between other glucocorticoid related complications and BMD or vertebral fracture in high dose glucocorticoid users (chi-square test).

Vertebral Fracture	Yes	No	p
Diabetes mellitus	26	134	
HbA1c, mg/dl*	7.68 ± 1.93	5.15 ± 0.66	< 0.01
BMD, g/cm^2	0.858 ± 0.149	0.850 ± 0.17	NS
Vertebral fracture, yes/no (%)	5/21 (19.2)	29/105 (21.6)	NS
Hyperlipidemia (cases)	95	65	
Total cholesterol, mg/dl*	283.2 ± 54.8	207.8 ± 23.0	< 0.01
BMD, g/cm^2	0.834 ± 0.176	0.876 ± 0.173	0.03
Vertebral fracture, yes/no (%)	23/72 (24.2)	11/54 (16.9)	NS

* Peak values after glucocorticoid therapy are shown. Patients whose value was > 220 mg/dl was defined to have hyperlipidemia. NS: not significant.

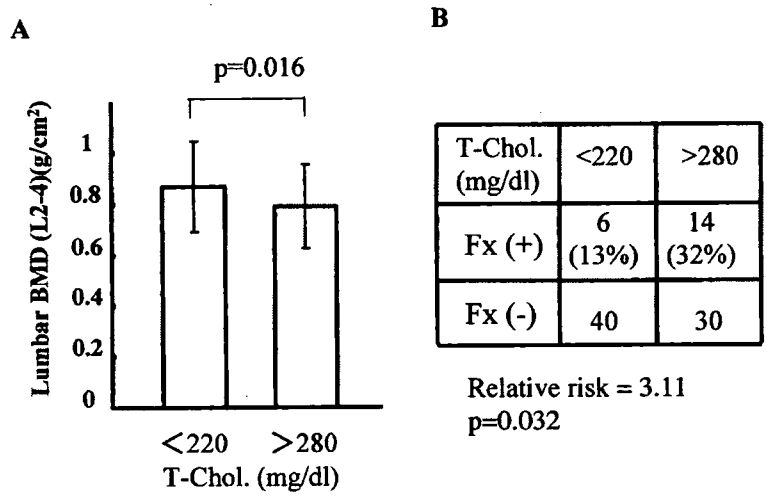

Figure 3. Influence of hyperlipidemia on lumbar BMD and vertebral fracture (Fx) in high dose glucocorticoid users. (A) Comparison of lumbar BMD between patients with high (> 280 mg/dl) and with normal (< 220 mg/dl) total cholesterol (T-Chol) values. (B) Comparison of the ratio of vertebral fracture between patients with high (> 280 mg/dl) and with normal (< 220 mg/dl) total cholesterol values. Chi-square analysis revealed that vertebral fracture was more frequent in patients with high total cholesterol level than in those with normal level (relative risk = 3.11, p = 0.032).

mean value of a large-scale Japanese study of primary osteoporosis by Orimo, *et al*, in which the cutoff value for osteoporosis was determined to be 70% of young adult mean[14]. There have been arguments about the difference of BMD threshold for fractures between postmenopausal users of glucocorticoids and nonusers. There are reports showing the BMD distribution of patients with vertebral fractures was similar for glucocorticoid users and nonusers[19,20]. On the other hand, other studies found that postmenopausal women taking glucocorticoids had a higher risk of fracture compared with nonusers, even at comparable levels of BMD[11,21]. Although our study was not designed to address this controversy, the relatively high BMD cutoff value, 80% of the young adult mean, for premenopausal women established in our study suggests that BMD alone may not be sufficient for predicting the risk of vertebral fracture for premenopausal users of glucocorticoids.

This notion is supported by our finding that premenopausal glucocorticoid users frequently experienced complications of vertebral fracture even when they registered normal BMD values. Vertebral fracture was seen in as many as 43% of premenopausal glucocorticoid users even when their BMD values were not particularly low (T score > –1). Recent guidelines from Europe and North America have been developed to establish intervention thresholds for glucocorticoid induced osteoporosis in patients with high BMD levels[22,23] or regardless of BMD level[24]. The recent guidelines of the American College of Rheumatology advocate intervention for all patients whose therapy calls for use of > 5 mg/day glucocorticoid for at least 3 months, and for

patients on a longterm glucocorticoid regimen with a BMD below a T score of −1.0[22]. Guidelines from the UK advocate an intervention threshold at a T score of −1.5 for patients who are scheduled to be given > 7.5 mg/day glucocorticoid for at least 6 months[23]. Our results suggest the need for developing a new therapeutic approach to prevent glucocorticoid induced osteoporosis in addition to starting antiresorptive therapy at high BMD thresholds.

Accumulating findings indicate that BMD is not the only factor that affects the risk of vertebral fracture[1,12,25]. One mechanism for the rapid onset of fracture risk could be osteocyte apoptosis, which leads to a deterioration of bone quality and a rapid increase in fracture risk[10]. Osteocyte apoptosis is prevalent in glucocorticoid induced osteoporosis[26]. The network of osteocytes is thought to detect microdamage to bone and be involved in bone repair remodeling. Therefore, osteocyte apoptosis together with glucocorticoid induced suppression of osteoblast generation could lead to growing micro-damage and a resultant increase in bone fragility. Thus, it is important to develop a new method to estimate bone fragility besides BMD measurement.

Another candidate factor that may contribute to the risk of osteoporosis from our study is hyperlipidemia. Our results showed that high total cholesterol (> 280 mg/dl) may be a risk factor for low BMD and vertebral fracture. There are reports of *in vitro* studies suggesting that low density lipoprotein oxidation products could promote osteoporosis by inhibiting osteoblast differentiation and by directing progenitor marrow stroma cells to undergo adipogenic instead of osteogenic differentiation[27,28]. Although these *in vitro* studies imply the possible involvement of lipid metabolism in the process of osteoporosis, there has been no report confirming the relationship of hyperlipidemia and glucocorticoid induced osteoporosis, and many clinical trials examining the efficacy of HMG-CoA reductase in preventing osteoporosis have had negative results. Therefore, further investigation is needed to establish a therapeutic strategy for preventing glucocorticoid induced osteoporosis in patients with hyperlipidemia.

Some reports stress the importance of daily glucocorticoid dose (mean) over cumulative glucocorticoid dose as an effective predictor of fracture[4,5,11], while others stress cumulative rather than daily glucocorticoid dose[6,7]. We detected no statistically significant difference between the occurrence of fracture and the mean daily glucocorticoid dose ($p = 0.483$) or cumulative glucocorticoid dose ($p = 0.794$), probably because of the limitation of our cross-sectional study and the limited numbers of patients with fracture. An important factor affecting our results may be differences in the use of antiresorptive drugs, especially bisphosphonates. This may be due partly to the Japanese legislative environment, since prophylactic use of drugs has not been allowed yet in the Japanese health insurance system. As this is a cross-sectional study, there are some limitations to interpreting our results. The onset of vertebral fracture is not predictable in prevalent fracture cases, and in these cases the influence of BMD may be different from that in incident fracture cases. To address these questions, we are now conducting a randomized cohort trial on patients who start glucocorticoid administration at a high dose, > 0.5 mg/kg.

Our findings support the hypothesis that treatment with glucocorticoids influences the occurrence of vertebral fracture by means of a mechanism independent of BMD. Moreover, it will be necessary to develop a new approach to assess and reduce the risk of vertebral fracture in premenopausal users of glucocorticoids.

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Elevated Levels of Soluble Fractalkine in Active Systemic Lupus Erythematosus

Potential Involvement in Neuropsychiatric Manifestations

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Objective. **To determine levels of the soluble form of the chemokine fractalkine (sFkn) and its receptor, CX_3CR1, in patients with systemic lupus erythematosus (SLE) with neuropsychiatric involvement (NPSLE) and in SLE patients without neuropsychiatric involvement, and to assess their relationship with disease activity and organ damage.**

Methods. **Levels of sFkn in serum and cerebrospinal fluid (CSF) were measured by enzyme-linked immunosorbent assay. Expression of Fkn and CX_3CR1 was quantified using real-time polymerase chain reaction. Surface expression of CX_3CR1 on peripheral blood mononuclear cells (PBMCs) was determined by flow cytometry. Disease activity and organ damage were assessed using the SLE Disease Activity Index (SLEDAI) and the Systemic Lupus International Collaborating Clinics/American College of Rheumatology (SLICC/ACR) Damage Index, respectively.**

Results. **Serum sFkn levels were significantly higher in patients with SLE than in patients with rheumatoid arthritis (RA) or healthy controls. In addition, significant correlations between serum sFkn levels and the SLEDAI, the SLICC/ACR Damage Index, anti–double-stranded DNA and anti-Sm antibody titers, immune complex levels (C1q), and serum complement levels (CH50) were observed. Expression of CX_3CR1 was significantly greater in PBMCs from patients with active SLE than in those from RA patients or healthy controls. Levels of sFkn were also significantly higher in CSF from untreated patients with newly diagnosed NPSLE than in SLE patients without neuropsychiatric involvement; treatment reduced both serum and CSF levels of sFkn in patients with SLE.**

Conclusion. **Soluble Fkn and CX_3CR1 may play key roles in the pathogenesis of SLE, including the neuropsychiatric involvement. Soluble Fkn is also a serologic marker of disease activity and organ damage in patients with SLE, and its measurement in CSF may be useful for the diagnosis of NPSLE and followup of patients with NPSLE.**

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by multiorgan damage with infiltration and sequestration of various leukocyte subpopulations, and by the presence of autoantibodies (1). Its etiology is known to involve dysregulation of the immune system, leading to a functional imbalance of T cell subsets, production of a wide range of autoantibodies, and polyclonal B cell activation. In addition, the importance of dysregulation of cytokine expression has been noted (2).

A variety of diffuse and focal neuropsychiatric symptoms often occur in patients with SLE. The features of this condition may include seizures, stroke, depression, psychosis, and cognitive disorders (3). Although the pathogenesis of neuropsychiatric SLE (NPSLE) has not been completely elucidated, a variety of clinical,

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laboratory, and radiographic findings are reportedly abnormal in some, but not all, SLE patients with central nervous system (CNS) complications, and the direct and indirect effects of several inflammatory mediators have been emphasized as possible contributors (4).

The chemokine fractalkine (Fkn; CX_3CL1) is synthesized as a type 1 transmembrane protein by endothelial cells (5). The soluble form of Fkn (sFkn) reportedly exerts a chemotactic effect on monocytes, natural killer (NK) cells, and T lymphocytes and acts via its receptor, CX_3CR1, as an adhesion molecule that is able to promote the firm adhesion of a subset of leukocytes to endothelial cells under conditions of physiologic flow (6). Notably, prominent expression of both Fkn and CX_3CR1 has been observed in the CNS (7). Thus, Fkn appears to possess immunoregulatory properties that affect inflammatory/immune cell–endothelial cell interactions and inflammatory responses.

The aim of the present study was to determine serum and CSF levels of sFkn and CX_3CR1 in SLE patients (those with and those without neuropsychiatric involvement) and to assess the relationship of these levels with disease activity and organ damage.

PATIENTS AND METHODS

Patients and samples of serum and CSF. A total of 67 serum samples were obtained from 53 patients with SLE (50 women and 3 men; mean ± SEM age 35.8 ± 1.8 years). In 14 patients, serum samples were collected during both the active and inactive phases of disease. All patients previously or currently fulfilled the American College of Rheumatology (ACR) revised criteria for the classification of SLE (8). Serum samples were also obtained from 91 patients with rheumatoid arthritis (RA) (71 women and 20 men; mean ± SEM age 65.3 ± 1.3 years) who fulfilled the 1987 revised ACR (formerly, the American Rheumatism Association) criteria for a diagnosis of RA (9), and from 28 healthy volunteers (16 women and 12 men; mean ± SEM age 34.4 ± 2.7 years). CSF from the lumbar spine was collected for the purpose of diagnosing NPSLE. For ethical reasons, CSF samples were not collected from SLE patients without any neuropsychiatric involvement or from healthy volunteers.

The SLE Disease Activity Index (SLEDAI) (10) was used to estimate general disease activity, and the Systemic Lupus International Collaborating Clinics (SLICC)/ACR Damage Index (11) was used to estimate organ damage.

Because of the difficulty in confirming neurologic diagnoses and of assigning cause to SLE, we defined NPSLE as the presence of at least 1 clinical feature of neuropsychiatric syndromes (3) and at least 1 of the following: pathologic findings on brain magnetic resonance imaging, diffusely abnormal results of brain single-photon–emission computerized tomography, severely abnormal results on a neuropsychiatric test, an elevated CSF IgG index, or increased interleukin-6 (IL-6) activity in the CSF (12).

Serum levels of specific autoantibodies, complement hemolysis activity (CH50), and immune complex (C1q) as well as albumin and IgG levels in both serum and CSF were determined in the clinical laboratory at our hospital. All human experiments were carried out in accordance with protocols approved by the Human Subjects Research Committee at our institution, and informed consent was obtained from all patients and volunteers.

Soluble Fkn levels. Soluble Fkn was quantified using a double ligand enzyme-linked immunosorbent assay (ELISA) that was a modification of an assay described previously (13). Monoclonal murine anti-human Fkn (4 μg/ml; Genzyme/Techne, Cambridge, MA) and biotinylated polyclonal goat anti-Fkn (0.25 μg/ml; Genzyme/Techne) served as the primary and the secondary antibodies, respectively. This ELISA detects the chemokine domain of human Fkn, and the sensitivity limit is ~150 pg/ml.

Flow cytometry. Flow cytometric analyses of CX_3CR1 expression on peripheral blood mononuclear cells (PBMCs) were carried out as previously described (14). PBMCs were obtained from heparinized venous blood from patients with SLE, patients with RA, and healthy volunteers and then labeled with the indicated primary antibody (anti-CD3–fluorescein isothiocyanate [FITC], anti-CD4–phycoerythrin [PE], anti-CD8–PE, and anti-CD14 [monocyte]–FITC; BD PharMingen, San Diego, CA), or rabbit anti-CX_3CR1 antibody (ProSci, Poway, CA), and then with a secondary antibody (biotin-conjugated anti-rabbit IgG) and a tertiary reagent (CyChrome-conjugated streptavidin; BD PharMingen). The fluorescence intensity was measured on a 3-color FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Isolation of total RNA, and real-time polymerase chain reaction (PCR). Total RNA extracted from PBMCs was reverse transcribed, and then real-time PCR was carried out in a LightCycler (Roche Diagnostics, Mannheim, Germany). To compare quantitative results between different samples, a dilution series of complementary DNA from unstimulated human umbilical vein endothelial cells and normal human PBMCs, which served as internal standards for Fkn and CX_3CR1, respectively, were loaded every time and assigned a value of 100 units. The primers used in the real-time PCR were as follows: for human CX_3CR1, 5′-AGCAGGCATGGAAGTGTTCT (sense) and 5′-GTTGTTTTGTGTGCATTGGG (antisense); for human Fkn, 5′-GCTGAGGAACCCATCCAT (sense) and 5′-GAGGCTCTGGTAGGTGAACA (antisense); for β-actin, which served as an internal control, 5′-CCCAAGGCCAACCGCGAGAAGAT (sense) and 5′-GTCCCGGCCAGCCAGGTCCAG (antisense).

Statistical analysis. Data are expressed as the mean ± SEM. Differences between groups were analyzed using the Mann-Whitney U test. Followup data were analyzed using Wilcoxon's test. The relationship between sFkn levels and the indicated parameters was evaluated using Spearman's rank correlation. P values less than 0.05 were considered significant.

RESULTS

Serum sFkn levels. We initially used ELISAs to assay the levels of sFkn in serum samples obtained from SLE patients with and those without neuropsychiatric

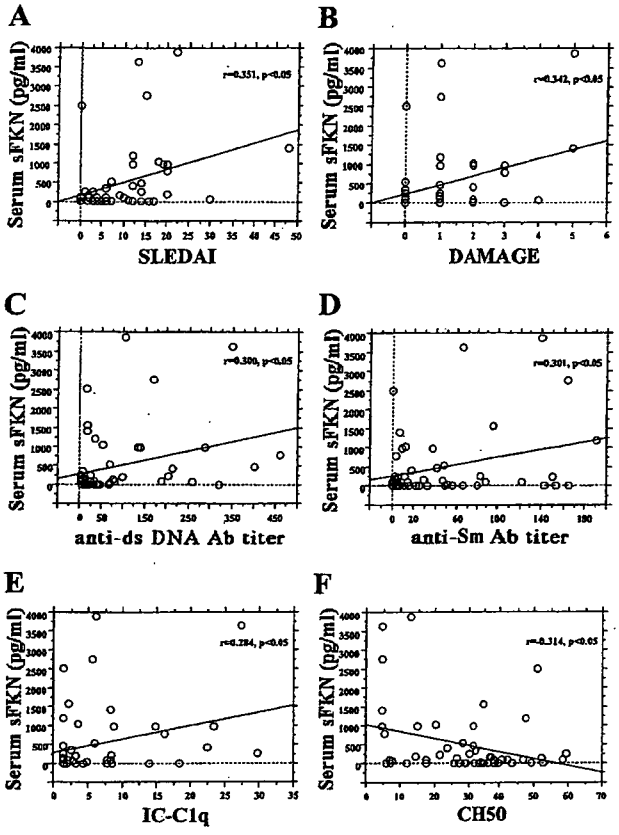

Figure 1. Correlation between serum levels of soluble fractalkine (sFkn) and various clinical parameters. The correlation between serum levels of sFkn (n = 67 samples) and the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (**A**), organ damage (Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index) (**B**), serum anti–double-stranded DNA (anti-dsDNA) antibody (Ab) titers (**C**), serum anti-Sm antibody titers (**D**), immune complex (IC-C1q) levels (**E**), and serum complement hemolysis activity (CH50) (**F**) in patients with SLE was examined. Serum levels of sFkn were assessed by enzyme-linked immunosorbent assay. Each point represents an individual SLE patient.

involvement (n = 67 samples), patients with RA (n = 91), and healthy controls (n = 28). Serum levels of sFkn were significantly higher in patients with SLE (mean ± SEM 452.7 ± 118.0 pg/ml) than in either patients with RA (mean ± SEM 225.2 ± 53.2 pg/ml; $P < 0.05$) or healthy controls (mean ± SEM 3.2 ± 3.2 pg/ml; $P < 0.01$). We then examined the relationship between serum levels of sFkn and disease activity, organ damage, and the indicated serologic parameters (Figure 1). We observed that serum levels of sFkn were correlated with both disease activity as measured by the SLEDAI (r = 0.351, $P < 0.05$) (Figure 1A) and organ damage as measured by the SLICC/ACR Damage Index (r = 0.342, $P < 0.05$) (Figure 1B) and were also positively correlated with anti–double-stranded DNA (anti-dsDNA) antibody titers (r = 0.300, $P < 0.05$), anti-Sm antibody titers (r = 0.301, $P < 0.05$), and immune complex C1q levels (r = 0.284, $P < 0.05$) (Figures 1C–E) and were negatively correlated with CH50 (r = −0.314, $P < 0.05$) (Figure 1F).

Expression of Fkn and CX_3CR1 messenger RNA (mRNA) and cell-surface expression of CX_3CR1. To better understand the dysregulation of Fkn/CX_3CR1 expression that occurs in SLE, we examined their expression profiles. CX_3CR1 mRNA was more strongly expressed in PBMCs from SLE patients than in those from patients with RA or healthy controls (Figure 2A). In contrast, Fkn expression in PBMCs from all 3 groups was markedly weak, and no significant difference between the groups was observed (results not shown). To examine in more detail the phenotype of cells expressing CX_3CR1, we used flow cytometry to analyze the protein expression of CX_3CR1 in peripheral blood–specific cell populations from SLE patients with active or inactive disease, patients with RA, and healthy controls (Figure 2B). Although both the intensity of CX_3CR1 expression on macrophages (results not shown) and the relative number of affected cells were slightly higher in patients with active SLE than in patients with inactive SLE or healthy controls, the expression of CX_3CR1 protein was most pronounced on CD4+,CD3+ T cells and CD8+,CD3+ T cells from a patient with untreated active SLE.

Neuropsychiatric manifestations and CSF levels of sFkn. Because Fkn has been detected in the nervous system (7), we hypothesized that it may also be involved in the pathogenesis of NPSLE. To test this hypothesis, we first assayed the sFkn levels in CSF from untreated patients with newly diagnosed active SLE, with or without neuropsychiatric involvement. As shown in Figure 3, levels of sFkn in CSF samples from all but 1 SLE patient without neuropsychiatric involvement (non-NPSLE) were relatively low (n = 6, mean ± SEM 186.3 ± 177.1 pg/ml) compared with those in patients with NPSLE (n = 6, mean ± SEM 842.7 ± 190.0 pg/ml). Notably, in contrast with the results observed in CSF, no significant difference in serum sFkn levels was observed between untreated patients with newly diagnosed NPSLE (n = 6, mean ± SEM 467.4 ± 24.0 pg/ml) and SLE patients without overt neuropsychiatric involvement (n = 6, mean ± SEM 400.3 ± 182.0 pg/ml). In addition, there were no significant differences in any serologic para-

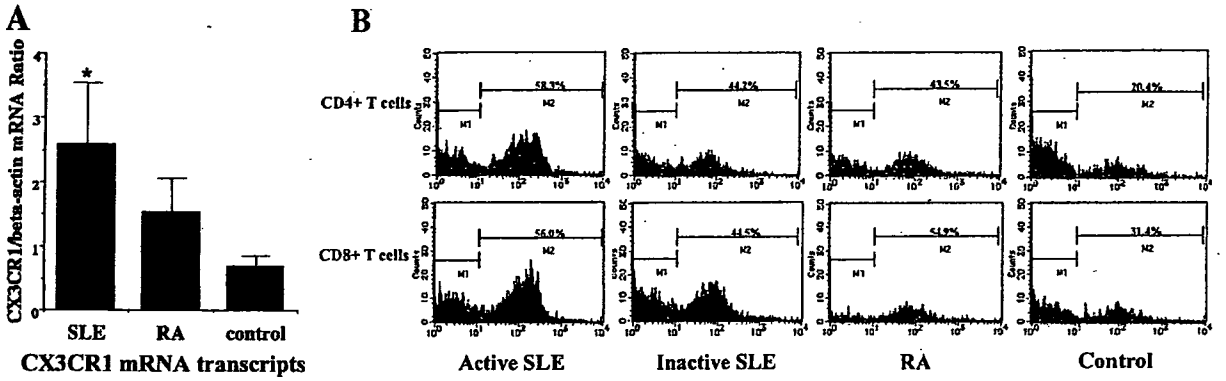

Figure 2. CX_3CR1 expression in peripheral blood mononuclear cells (PBMCs). **A,** Total RNA was isolated from PBMCs obtained from 21 patients with systemic lupus erythematosus (SLE), 30 patients with rheumatoid arthritis (RA), and 10 healthy controls, after which the cDNA was reverse transcribed, and real-time polymerase chain reaction was carried out. Levels of CX_3CR1 mRNA are expressed as the mean and SEM units. * = $P < 0.05$ versus RA and control. **B,** PBMCs obtained from untreated patients with newly diagnosed SLE (active), treated patients with inactive SLE, patients with RA, and healthy controls were labeled with anti-CD3+, anti-CD4+, anti-CD8+, or anti-CX_3CR1 antibody. CX_3CR1 expression on gated cells (CD4+,CD3+ T cells; CD8+,CD3+ T cells) was assayed by 3-color flow cytometry. Samples obtained from patients with SLE were followed up. M1 = background intensity of isotype-matched control staining. M2 = percent of CX_3CR1-positive cells. Histograms are representative of 3 independent experiments.

meters between patients with NPSLE and SLE patients without neuropsychiatric involvement. Moreover, the IL-6 concentration was shown to be elevated in the CSF of some patients with NPSLE (12), but we found no significant correlation between CSF levels of sFkn and IL-6 activity in the CSF ($P = 0.32$).

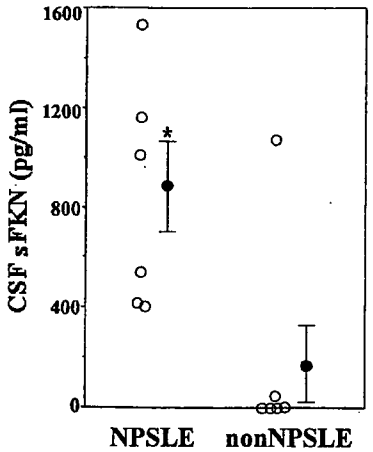

Figure 3. Levels of soluble fractalkine (sFkn) in cerebrospinal fluid (CSF). Samples of CSF were obtained from 6 untreated patients with newly diagnosed neuropsychiatric systemic lupus erythematosus (NPSLE) and 6 SLE patients without neuropsychiatric involvement (non-NPSLE; of these 6 patients who did not fulfill our criteria for NPSLE, 4 described having mild headache, and 2 had mild mood disorder). Soluble Fkn levels were determined by enzyme-linked immunosorbent assay. Each point represents an individual patient. Bars show the mean ± SEM. * = $P < 0.05$ versus non-NPSLE.

Because of the small number of samples examined, we were unable to determine the statistical significance of differences in CSF sFkn levels among patients with any particular neuropsychiatric manifestation. However, when neuropsychiatric manifestations were classified as either diffuse CNS disease (n = 2), which included psychosis, mood disorder, cognitive dysfunction, and acute states of confusion, or as focal CNS disease (n = 4), which included cerebrovascular disease, demyelinating syndrome, headache, aseptic meningitis, seizures, or myelopathy (3), sFkn levels tended to be higher in CSF from patients with focal disease (mean ± SEM 1,029.0 ± 234.1 pg/ml versus 470.0 ± 69.0 pg/ml in patients with diffuse disease).

Followup studies of the effect of treatment on CSF and serum sFkn levels. Figure 4 summarizes the results of followup studies of serum levels of sFkn in 14 patients with SLE (with or without neuropsychiatric manifestations) before and 2–3 months after treatment with glucocorticoids and other immunosuppressive drugs (12 patients received glucocorticoids alone, and 2 patients received glucocorticoids plus cyclophosphamide or cyclosporin A). Notably, serum sFkn levels in patients with active SLE were significantly diminished following successful treatment and clinical improvement (mean 559.4 pg/ml in patients with active disease versus 102.1 pg/ml in patients inactive disease). Although the mean reduction in the CSF

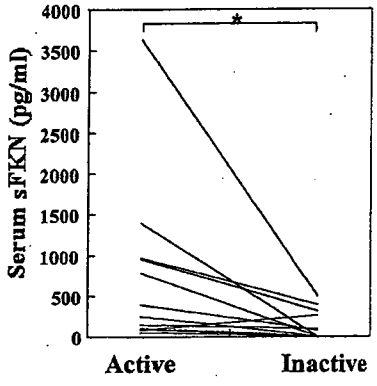

Figure 4. Followup measurements of soluble fractalkine (sFkn) levels in serum from patients with systemic lupus erythematosus (SLE), with or without neuropsychiatric involvement. Paired samples of serum were obtained from 14 patients with SLE (with or without neuropsychiatric involvement) at the time of active disease (newly diagnosed, untreated) and after treatment (inactive disease). Each line represents an individual patient. * = $P < 0.05$.

of 4 patients with NPSLE was quite pronounced (from 877.3 pg/ml to 155.3 pg/ml), it did not reach statistical significance.

DISCUSSION

In the present study, we showed that serum sFkn levels were significantly higher in patients with SLE than in patients with RA or healthy controls and were positively correlated with disease activity, organ damage, anti-dsDNA and anti-Sm antibody titers, and immune complex levels and were negatively correlated with CH50 activity. In addition to the increased expression of sFkn itself, increased expression of its receptor, CX_3CR1, was also detected, especially on CD4+ and CD8+ T cells from patients with active SLE. Finally, levels of sFkn in the CSF were elevated in patients with NPSLE, and both serum and CSF levels of sFkn were reduced by successful treatment with glucocorticoids and other immunosuppressive drugs.

This study is the first to demonstrate increases in sFkn levels in the peripheral blood and CNS of patients with active SLE and patients with NPSLE, respectively. Recent evidence indicates that receptor expression determines the spectrum of action of chemokines in Th1 and Th2 cells. Indeed, Fraticelli et al recently reported that CX_3CR1 was preferentially expressed in Th1 cells, and that Th1 cells, but not Th2 cells, respond to Fkn (15). Furthermore, Fkn also acts via CX_3CR1 as an adhesion molecule and as a chemoattractant, recruiting monocytes, NK cells, and T lymphocytes to endothelial cells. Thus, Fkn likely plays multiple roles in the development of SLE, via Th1 cell–endothelial cell interactions.

Intracranial increases in a variety of cytokines, including IL-6, have been observed in patients with NPSLE (12). This suggests that these various proinflammatory and antiinflammatory cytokines all play specific roles during the progression of NPSLE. In the present study, however, we observed no significant correlation between the levels of sFkn and IL-6 in the CSF of patients with NPSLE, which may indicate that the expression of Fkn and IL-6 is differentially regulated by these 2 mediators during the evolution of the neuropsychiatric manifestations in patients with SLE. Furthermore, we observed that patients with focal neuropsychiatric manifestations had higher CSF levels of sFkn than did those with diffuse disease. These findings are not consistent with the results reported by Erichsen et al (16), who found that sFkn levels in the CSF of human immunodeficiency virus type 1 (HIV-1)–infected patients with cognitive impairment (diffuse disease) were significantly higher than those in HIV-1–infected patients without cognitive impairment. It would be interesting to know whether this difference reflects a difference in the underlying mechanism of the pathogenesis of NPSLE and HIV-induced encephalopathy, and the extent to which Fkn participates in those processes.

In healthy individuals, surface expression of CX_3CR1 has been demonstrated in NK cells, monocytes, and effector T cells (17). CX_3CR1 is also expressed on CD4+ and CD8+ T cells in patients with RA (18). Consistent with those findings, we observed increased expression of CX_3CR1 mainly on CD4+ and CD8+ T cells in patients with active SLE. Moreover, T cell expression of CX_3CR1 was significantly reduced by treatment that diminished disease activity. Although there have been few studies of the expression and regulation of CX_3CR1 under pathologic conditions, it is noteworthy that CX_3CR1 expression on immune cells parallels the sFkn levels, suggesting that CX_3CR1 mediates activation of recruited inflammatory cells, especially CD4+ and CD8+ T cells, during active SLE.

In conclusion, sFkn and CX_3CR1 may play important roles in the pathogenesis of SLE, including the neuropsychiatric involvement. Soluble Fkn is also a serologic marker of disease activity and organ damage in patients with SLE, and its measurement in CSF may be useful for the diagnosis of NPSLE and the followup of patients with NPSLE.

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Differential effects of IFN-α on the expression of various T_H2 cytokines in human $CD4^+$ T cells

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Background: In both human subjects and mice, T helper cells are classified into 2 subsets, T_H1 and T_H2 cells, on the basis of the cytokines they produce. Although IFN-α has been shown to enhance human T_H1 responses, its influences on human T_H2 responses have not yet been fully characterized. In addition, the mechanism for induction of T_H1 responses by IFN-α has not been fully delineated.

Objective: The present study was undertaken to explore the direct effects of IFN-α on the expression of various cytokines in human $CD4^+$ T cells with a system using immobilized anti-CD3, which permits activation of $CD4^+$ T cells in the complete absence of accessory cells.

Methods: Highly purified $CD4^+$ T cells obtained from healthy donors were stimulated with immobilized anti-CD3 with or without IFN-α and IL-12 in the complete absence of accessory cells. The production of cytokines was estimated by means of ELISA. The expression of mRNA for various cytokines, as well as transcription factors, was evaluated by using quantitative PCR.

Results: IFN-α enhanced IL-4 protein and mRNA expression in immobilized anti-CD3–stimulated $CD4^+$ T cells, irrespective of the presence of IL-12, whereas IFN-α suppressed the expression of IL-5 and IL-13. Of note, IFN-α enhanced the expression of mRNA for c-Maf, T-bet, and Fox-P3, irrespective of the presence of IL-12, but not that for GATA-3, in anti-CD3–stimulated $CD4^+$ T cells.

Conclusion: These results indicate that IFN-α enhances the induction of T_H1 responses through upregulation of T-bet mRNA expression, as well as the induction of T_H2 responses through upregulation of c-Maf mRNA expression, followed by IL-4 expression. Moreover, the data also suggest that IFN-α might suppress the expression of IL-5 and IL-13 in differentiated T_H2 cells. (J Allergy Clin Immunol 2005;116:205-12.)

Key words: Human, T_H1, T_H2, IL-4, IL-5, IL-13, c-Maf, GATA-3, T-bet, Fox-P3

Abbreviations used
IL-12Rβ2: IL-12 receptor β2
NK: Natural killer

In both human subjects and mice, activated $CD4^+$ T cells can be classified into 2 subsets, T_H1 and T_H2 cells, on the basis of the cytokines they produce.[1-4] Thus T_H1 cells produce IFN-γ and IL-2, which are involved in cell-mediated immune responses, whereas T_H2 cells produce mainly IL-4, IL-5, and IL-13, which are involved in humoral immune responses.[5,6] IL-4 has a major role in B-cell activation and isotype switching, particularly in IgE production.[7] On the other hand, IL-5 activates mature eosinophils, prolongs their survival, and contributes to their accumulation at sites of inflammation.[8] In T_H2 cells the transcription factors GATA-3 and c-Maf are selectively expressed and have been shown to regulate T_H2 cytokine expression.[9,10] Thus c-Maf is required for the expression of IL-4,[11] whereas GATA-3 is involved in the expression of IL-5 and IL-13.[12,13]

IFN-α presents potent antiviral actions, as well as immunoregulatory activities, including enhancement of cytotoxic activity of T cells and natural killer (NK) cells.[14] IFN-α has been shown to enhance human T_H1 responses, which is reflected by IFN-γ production in the presence of accessory cells with stimulation by PHA.[15-17] We have recently revealed that IFN-α by itself did not enhance IFN-γ production or mRNA expression in anti-CD3–stimulated human $CD4^+$ T cells in the absence of accessory cells or exogenous IL-12.[18] Consistently, IFN-α enhanced IL-12 receptor β2 (IL-12Rβ2) mRNA expression in $CD4^+$ T cells.[18] It is therefore indicated that the induction of human T_H1 responses by IFN-α requires the presence of IL-12.[18] As for human T_H2 responses, it was previously shown that IFN-α inhibits IL-5 production and mRNA expression in $CD4^+$ T cells.[19] However, the effects of IFN-α on the production of IL-4 in human $CD4^+$ T cells have been uncertain, possibly because of contaminating accessory cells or other supplemental cell lines to cross-link CD3 molecules through soluble anti-CD3. In addition, IL-13 is one of the T_H2 cytokines that has very similar biologic actions of IL-4.[20] Although the regulation of IL-13 production is pivotal in the function of T_H2 cells, the effects of IFN-α on the production of IL-13 have not been determined. The current studies were therefore undertaken to explore the direct effects of IFN-α on the expression of the T_H2 cytokines IL-4, IL-5, and IL-13 in activated human $CD4^+$ T cells by using a system with immobilized anti-CD3, which permits stimulation of T cells in the complete absence of accessory cells or other supplemental cell lines. Special attention was paid to the effects of IFN-α on the expression of mRNA for a variety of transcription factors that regulate the polarization of

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T_H1 and T_H2 cells. The results demonstrate that IFN-α suppresses the expression of IL-5 and IL-13 and enhances the expression of IL-4 in $CD4^+$ T cells. More importantly, IFN-α enhanced the expression of mRNA for c-Maf, T-bet, and Fox-P3, but not for GATA-3, in $CD4^+$ T cells. The data indicate that IFN-α exerts a variety of effects on human T_H1 and T_H2 responses through regulation of mRNA for various transcription factors.

METHODS

mAbs and reagents

Anti-CD3 mAb 64.1 (an IgG2a mAb directed at the CD3 molecule on mature T cells) was a gift of Dr P. E. Lipsky (National Institute of Health, Bethesda, Md). Recombinant human IL-12 was purchased from PeproTech (Rocky Hill, NJ). Recombinant human IFN-α2a was a gift of Nippon Roche (Tokyo, Japan).

Culture medium

RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 100 U/mL penicillin G, 100 μg/mL streptomycin, 0.3 mg/mL L-glutamine, and 10% FBS (Life Technologies) was used for all cultures.

Cell preparation

PBMCs were obtained from healthy adult volunteers by means of centrifugation of heparinized venous blood over sodium diatrizoate-Ficoll gradients (Histopaque; Sigma Chemical Co, St Louis, Mo). PBMCs were depleted of monocytes and NK cells by means of incubation with 5 mM L-leucine methyl ester HCl (Sigma) in serum-free RPMI 1640, as described elsewhere.[21] T cells were obtained from the treated cell population by rosetting with neuraminidase-treated sheep red blood cells, as previously described.[22] Purified $CD4^+$ T cells were further prepared by means of positive selection with anti-CD4 microbeads and MACS (Miltenyi Biotec, Auburn, Calif). The $CD4^+$ T-cell population obtained in this manner contained less than 0.1% esterase-positive cells, less than 0.1% NK cells, less than 0.1% $CD19^+$ cells, and greater than 96% $CD4^+$ T cells.

Cell cultures

Anti-CD3 mAb 64.1 was diluted in RPMI 1640 (2 μg/mL), and 50 μL was placed in each well of 96-well flat-bottomed microtiter plates (no. 3596; Costar, Cambridge, Mass) and incubated at room temperature for 1 hour.[21] The wells were then washed once with culture medium to remove nonadherent mAb before the cells were added. Purified $CD4^+$ T cells (2×10^5/well) were cultured in wells with immobilized anti-CD3 with or without IFN-α (1×10^5 IU/mL) and IL-12 (10 ng/mL). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

RNA isolation and real-time quantitative PCR

Total RNA was isolated from cultured cells with Trizol reagent (Life Technologies), according to the manufacturer's application protocol, and quantified spectrophotometrically. cDNA samples were prepared from 1 μg of total RNA by using the SuperScript reverse transcriptase preamplification system (Life Technologies) with oligo (dT) primer and subjected to real-time quantitative PCR.

Real-time quantitative PCR was performed with the LightCycler rapid thermal cycler system (Roche Diagnostics Ltd, Lewes, United Kingdom), with ready-made primer sets for human IFN-γ, IL-4, IL-5, IL-12Rβ2, GATA-3, T-bet, Fox-P3, or β-actin (LightCycler-Primer Set; Roche Diagnostics GmbH, Heidelberg, Germany) and LightCycler-Fast Start DNA Master SYBR Green I (Roche Diagnostics Ltd). The primers for human c-Maf were designed as follows: forward, 5′-GGTCAGCAAGGAGGAGGT-3′; reverse, 5′-TCTCCTGCTTGAGGTGGTC-3′. PCR reaction condition was identical for all genes except for c-Maf (shown in parentheses): incubation at 95°C for 10 minutes, followed by 35 cycles (40 cycles) of 95°C for 10 seconds, 68°C (60°C for c-Maf) for 10 seconds, and 72°C for 16 seconds (6 seconds for c-Maf). Melting-curve analysis was then carried out to confirm the quality of the performance of the PCR by using 1 cycle of 95°C for 0 seconds and 58°C for 10 seconds (70°C for 15 seconds for c-Maf), with continuous increase to 95°C (rate, 0.1°C/s), followed by cooling at 40°C for 30 seconds. A standard curve was generated in each experiment by using a standard solution in each primer set, and quantitative analysis was performed with LightCycler Software version 3.5. All results were calibrated to the copy number (copies per microliter) of β-actin from each cDNA sample.

Measurement of IL-4, IL-5, and IL-13

IL-4 and IL-13 contents in the supernatants were measured with ELISA kits (Cytoscreen; BioSource International, Camarillo, Calif). The detection limits of the assays were approximately 2.0 and 12.0 pg/mL for IL-4 and IL-13, respectively. The assay is specific for natural and recombinant human IL-4 and IL-13. IL-5 contents in the supernatants were measured with a Human IL-5 ELISA development kit (PeproTech). The detection limit of the assay was approximately 2.0 pg/mL IL-5.

Statistical analysis

The results were analyzed for statistical significance by using the Wilcoxon signed-rank test.

RESULTS

The induction of T_H1 responses in immobilized anti-CD3–activated $CD4^+$ T cells by IFN-α totally depends on the presence of IL-12

We have previously shown that IFN-α enhanced IFN-γ production and mRNA expression only in the presence of IL-12.[18] Initial experiments were carried out to reexamine these direct effects of IFN-α on IFN-γ mRNA expression in activated human $CD4^+$ T cells. As summarized in Table I, IFN-α did not enhance IFN-γ mRNA expression of immobilized anti-CD3–activated $CD4^+$ T cells throughout the cultures, whereas IL-12 significantly enhanced this expression. Of note, IFN-α significantly enhanced IFN-γ mRNA expression in the presence of exogenous IL-12, but not in the absence of IL-12, as early as 3 hours of culture. The results therefore confirm the conclusion that upregulation of the expression of IFN-γ mRNA by IFN-α totally depends on the presence of IL-12.[18] Of note, IFN-α enhanced the expression of IL-12Rβ2 mRNA in anti-CD3–activated $CD4^+$ T cells, irrespective of the presence of IL-12 as early as 3 hours of culture (Table I), as is consistent with the results of a previous study.[18] Taken together, these data suggest that the induction of T_H1 responses by IFN-α is mediated through upregulation of the expression of functional IL-12R, although the precise

TABLE I. Effects of IFN-α and IL-12 on the expression of mRNA for IFN-γ and IL-12Rβ2 in immobilized anti-CD3–activated $CD4^+$ T cells

mRNA	Incubation	× 10^{-2} to β-actin mRNA copies (mean ± SD)			
		Nil	IFN-α	IL-12	IFN-α + IL-12
IFN-γ	3 h	9.8 ± 4.6	10.7 ± 5.1	14.6 ± 6.8*	20.9 ± 6.0*†
	24 h	6.9 ± 3.8	6.4 ± 3.1	27.8 ± 15.0*	38.4 ± 14.7*†
IL-12Rβ2	3 h	0.091 ± 0.072	0.473 ± 0.302*	0.133 ± 0.122*	0.613 ± 0.348*†
	24 h	1.017 ± 0.722	2.497 ± 1.149*	1.728 ± 1.279*	3.673 ± 2.187*†

$CD4^+$ T cells (2×10^5/well) from 6 healthy individuals were cultured in wells with immobilized anti-CD3 (mAb 64.1, 100 ng/well) with or without IFN-α (1×10^5 IU/mL) and IL-12 (10 ng/mL). After 3 or 24 hours of incubation, total RNA was isolated, and real-time quantitative PCR was performed with specific primers for IFN-γ, IL-12Rβ2, and β-actin. All results were calibrated to the copy number of β-actin (copies per microliter) from each cDNA sample.
*$P < .05$ compared with cultures without cytokines *(Nil)*.
†$P < .05$ compared with cultures with IL-12.

mechanism for the upregulation of IL-2R2 mRNA by IFN-α remains unclear.

Differential effects of IFN-α on the expression of T_H2 cytokines in anti-CD3–activated $CD4^+$ T cells

It was previously shown that IFN-α inhibits IL-5 production and mRNA expression in human $CD4^+$ T cells.[19] However, the effects of IFN-α on the production of IL-4 in human $CD4^+$ T cells have been unclear in these studies.[19] It was possible that contaminating accessory cells or supplemental cells to facilitate cross-linkage of CD3 with soluble anti-CD3 might influence the results.[19] The next experiments therefore compared the direct effects of IFN-α on the production of the T_H2 cytokines IL-4, IL-5, and IL-13 in immobilized anti-CD3–activated $CD4^+$ T cells in the complete absence of accessory cells or other supplemental cells. As shown in Fig 1, the production of IL-4 appeared to reach its peak at 24 hours of culture, whereas that of IL-5 and IL-13 markedly increased between 24 and 72 hours. More importantly, IFN-α appeared to enhance the production of IL-4 as early as 24 hours of culture in a dose-dependent manner. By contrast, IFN-α seemed to decrease the production of IL-5 and IL-13 at 72 hours of culture.

The next experiments were carried out to confirm the effects of IFN-α on the expression of IL-4, IL-5, and IL-13 in anti-CD3–stimulated $CD4^+$ T cells. In accordance with previous studies,[19] IFN-α markedly suppressed IL-5 production at 72 hours of culture and IL-5 mRNA expression at 24 hours of culture, irrespective of the presence of IL-12 (Table II). Of note, IFN-α also significantly suppressed the production of IL-13 by $CD4^+$ T cells stimulated with immobilized anti-CD3 for 24 and 72 hours, irrespective of the presence of IL-12 (Table III). The data therefore indicate that IFN-α suppresses the expression of IL-5, as well as that of IL-13.

In contrast with IL-5 and IL-13, the production of IL-4 was increased very modestly between 24 and 72 hours of culture (Fig 1). More importantly, IFN-α significantly enhanced IL-4 production and mRNA expression of anti-CD3–activated $CD4^+$ T cells, irrespective of the presence of IL-12 (Table IV). The results indicate that IFN-α by itself promotes the expression of IL-4 protein and mRNA in spite of its enhancing effects on IL-12Rβ2 mRNA expression.

Effects of IFN-α on the expression of mRNA for a variety of transcription factors in anti-CD3–activated $CD4^+$ T cells

It has been revealed that the transcription factors c-Maf and GATA-3 are expressed exclusively in T_H2 cells and regulate T_H2 cytokine expression.[9,10] Thus c-Maf is required for the expression of IL-4,[11] whereas GATA-3 is critical for the expression of IL-5.[12] It was therefore possible that the effects of IFN-α on the mRNA expression of IL-4 and IL-5 in anti-CD3–activated $CD4^+$ T cells might result from changes in the expression of these transcription factors. On the other hand, previous studies have demonstrated that T-bet plays a critical role in the induction of T_H1 responses.[23] In addition, recent studies have demonstrated that Fox-P3 is required for the development of $CD4^+CD25^+$ regulatory T cells.[24] To examine the effects of IFN-α on the expression of mRNA for these transcription factors, total RNA was isolated from cultured cells, and real-time quantitative PCR was performed with specific primers for c-Maf, GATA-3, T-bet, Fox-P3, and β-actin.

As shown in Fig 2, IFN-α enhanced the expression of mRNA for c-Maf, T-bet, and Fox-P3 in a dose-response manner, whereas it did not appear to affect the expression of GATA-3 mRNA in $CD4^+$ T cells stimulated with immobilized anti-CD3 for 3 hours. Consistently, as can be seen in Fig 3, IFN-α significantly enhanced the expression of mRNA for c-Maf, T-bet, and Fox-P3 in anti-CD3–activated $CD4^+$ T cells, irrespective of the presence of IL-12, whereas IFN-α did not significantly affect the expression of GATA-3 mRNA. Of note, IL-12 also significantly upregulated the expression of mRNA for T-bet and Fox-P3. IFN-α further enhanced their expression in the presence of IL-12. These results suggest that IFN-α might enhance the production of IL-4 in anti-CD3–activated $CD4^+$ T cells by upregulating the expression of c-Maf mRNA. Moreover, the data indicate that IFN-α induces T_H1 responses through upregulation of T-bet. Finally, it is unlikely that the suppression of the expression of IL-5 and IL-13 by IFN-α might be accounted for by the downregulation of GATA-3 mRNA expression. Because

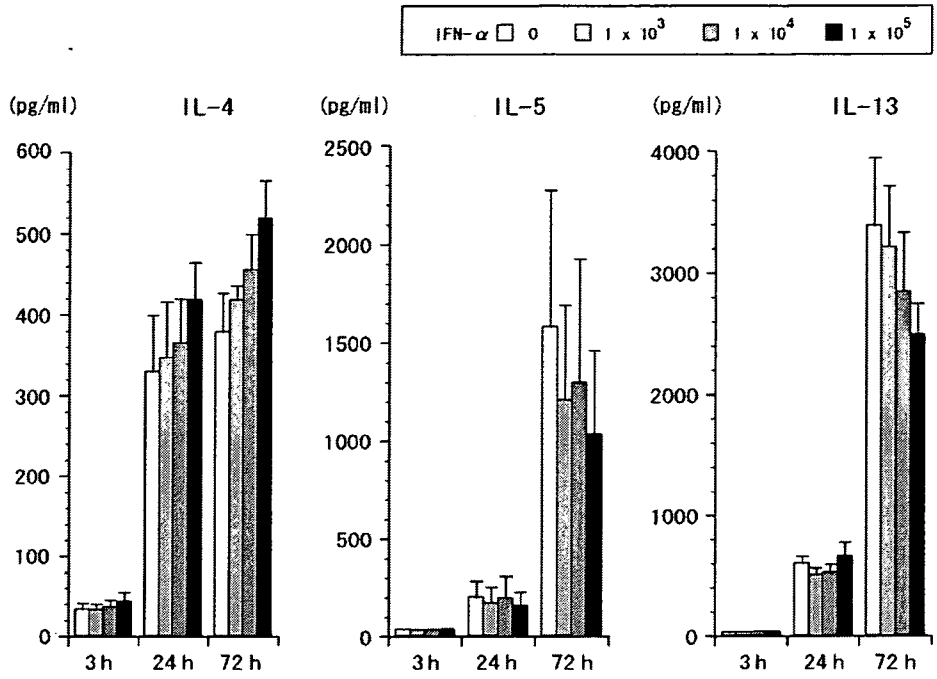

FIG 1. Effects of IFN-α on the production of IL-4, IL-5, and IL-13 by immobilized anti-CD3–activated $CD4^+$ T cells. $CD4^+$ T cells (2×10^5/well) were cultured in wells with immobilized anti-CD3 (mAb 64.1, 100 ng/well) with various concentrations of IFN-α. After 3, 24, or 72 hours of incubation, the supernatants were assayed for IL-4, IL-5, and IL-13 contents by means of ELISA. Mean values of 2 independent experiments are shown. *Error bars* represent the SD of 2 independent experiments.

TABLE II. Effects of IFN-α and IL-12 on IL-15 protein production and mRNA expression in immobilized anti-CD3–activated $CD4^+$ T cells

IL-13 production, pg/mL (mean ± SD)	IL-5 production, pg/mL (mean ± SD)	IL-5 mRNA (mean ± SD)
Nil	77.3 ± 52.3	5.183 ± 5.323
IFN-α	16.2 ± 26.5*	1.402 ± 1.671*
IL-12	87.2 ± 61.8	4.510 ± 3.995
IFN-α + IL-12	20.4 ± 11.6*†	1.643 ± 1.400*†

$CD4^+$ T cells (2×10^5/well) from 6 healthy individuals were cultured in wells with immobilized anti-CD3 (mAb 64.1, 100 ng/well) with or without IFN-α (1×10^5 IU/mL) and IL-12 (10 ng/mL). After 72 hours of incubation, the supernatants were assayed for IL-5 contents by means of ELISA. After 24 hours of incubation, total RNA was isolated, and real-time quantitative PCR was performed with specific primers for IL-5 and β-actin. All results were calibrated to the copy number of β-actin (copies per microliter) from each cDNA sample.

*$P < .05$ compared with cultures without cytokines *(Nil)*.

†$P < .05$ compared with cultures with IL-12.

IFN-α also enhanced Fox-P3 and T-bet mRNA expression, it is more likely that induction of T_H1-like regulatory cells that express Fox-P3[25] might be involved in suppression of the expression of IL-5 and IL-13.

DISCUSSION

Previous studies have reported that type 1 interferons (IFN-α/β) act directly on human, but not mouse, T cells to drive T_H1 development, bypassing the need for IL-12–induced signaling.[15] However, our previous and present studies demonstrated that IFN-α did not affect IFN-γ protein and mRNA expression in $CD4^+$ T cells unless IL-12 was present, confirming that IFN-α by itself might not be sufficient for the optimal induction of T_H1 responses.[18] It should be noted that previous studies explored IFN-α–induced T_H1 responses in the presence of accessory cells with stimulation by PHA.[15-17] Because accessory cells produce IL-12, it is likely that the induction of T_H1 responses by IFN-α in PHA-stimulated cultures might be mediated by cooperative actions of IFN-α and IL-12. In fact, no IL-12 could be detected in the culture supernatants of anti-CD3–activated $CD4^+$ T cells in our system.[18] These findings confirm that the presence of IL-12 is essential for the upregulation of IFN-γ expression by IFN-α. Consistently, we and others have demonstrated that IFN-α enhances the expression of IL-12Rβ2 mRNA in anti-CD3–activated $CD4^+$ T cells.[16,18] It was therefore most likely that IFN-α–induced T_H1 responses observed in the previous studies[15-17] might be mediated through upregulation of the responsiveness to IL-12 secreted from accessory cells. In fact, a recent study also suggests that IFN-α might enhance IFN-γ production in human T cells through IL-12–dependent mechanisms.[26]

We have shown that IFN-α enhanced the expression of T-bet mRNA in immobilized anti-CD3–activated $CD4^+$ T cells. In this regard it has recently been disclosed that IL-27 and IFN-α activate signal transducer and activator

TABLE III. Effects of IFN-α and IL-12 on IL-13 production by immobilized anti-CD3–activated $CD4^+$ T cells

	IL-13 production, pg/mL (mean ± SD)			
Incubation	**Nil***	**IFN-α**	**IL-12**	**IFN-α + IL-12**
24 h	137.6 ± 131.5	71.5 ± 59.5*	113.1 ± 88.5	62.4 ± 45.6*†
72 h	1278.8 ± 640.8	650.9 ± 429.0*	1310.8 ± 797.6	685.0 ± 496.0*†

$CD4^+$ T cells (2×10^5/well) from 7 healthy individuals were cultured in wells with immobilized anti-CD3 (mAb 64.1, 100 ng/well) with or without IFN-α (1×10^5 IU/mL) and IL-12 (10 ng/mL). After 24 or 72 hours of incubation, the supernatants were assayed for IL-13 contents by means of ELISA.
*$P < .05$ compared with cultures without cytokines *(Nil)*.
†$P < .05$ compared with cultures with IL-12.

TABLE IV. Effects of IFN-α and IL-12 on IL-4 protein production and mRNA expression in immobilized anti-CD3–activated $CD4^+$ T cells

		Addition			
Assays	**Length of incubation**	**Nil**	**IFN-α**	**IL-12**	**IFN-α ± IL-12**
IL-4 protein production (pg/mL) (mean ± SD)	24 h	46.9 ± 42.3	75.1 ± 58.8*	53.5 ± 49.1	77.1 ± 57.3*†
	72 h	103.1 ± 69.4	121.2 ± 81.6*	121.4 ± 71.8*	143.4 ± 86.5*†
IL-4 mRNA expression ($\times 10^{-3}$ to β-actin mRNA copies) (mean ± SD)	3 h	2.172 ± 1.662	4.602 ± 2.718*	1.403 ± 0.835*	4.145 ± 2.445*†
	24 h	1.478 ± 1.052	1.943 ± 1.044*	1.213 ± 0.781*	1.688 ± 1.242†

$CD4^+$ T cells (2×10^5/well) from 8 healthy individuals were cultured in wells with immobilized anti-CD3 (mAb 64.1, 100 ng/well) with or without IFN-α (1×10^5 IU/mL) and IL-12 (10 ng/mL). After 24 or 72 hours of incubation, the supernatants were assayed for IL-4 contents by means of ELISA. After 3 or 24 hours of incubation, total RNA was isolated, and real-time quantitative PCR was performed with specific primers for IL-4 and β-actin. All results were calibrated to the copy number of β-actin (copies per microliter) from each cDNA sample.
*$P < .05$ compared with cultures without cytokines *(Nil)*.
†$P < .05$ compared with cultures with IL-12.

of transcription 1 and 3 to induce T-bet mRNA in naive T cells.[27] Induction of T-bet resulted in upregulation of IL-12Rβ2 on naive T cells.[27] It was thus possible that T-bet might act upstream of IL-12Rβ2 in early T_H1 differentiation. However, IFN-γ induced expression of T-bet, but not IL-12Rβ2, in naive T cells.[27] It is therefore most likely that T-bet and IL-12Rβ2 might be regulated by independent mechanisms. In addition, our data indicate that T-bet and IL-12Rβ2 are not sufficient for the induction of optimal T_H1 responses, although they are important for early T_H1 commitment.[27]

Several studies showed that the T_H2 cytokines IL-4, IL-5, and IL-13 are regulated by a coordinated mechanism.[28,29] On the other hand, a number of other studies showed evidence for differential regulation of the expression of IL-4 and IL-5 in murine and human T cells.[30-32] The results in the current studies have disclosed that IFN-α displays differential effects on the expression of these T_H2 cytokines. Thus IFN-α suppressed the expression of IL-5 and IL-13, whereas it enhanced the expression of IL-4 in anti-CD3–activated $CD4^+$ cells. Of note, in the report by Cousins et al,[29] the expression of IL-5 is well correlated with that of IL-13, but not with that of IL-4. Taken together, it is most likely that the expression of various T_H2 cytokines might be differentially regulated in $CD4^+$ T cells.

Some studies disclosed that IFN-α inhibited the differentiation of T_H2 cells producing IL-4 and IL-5 in bulk cultures of PBMCs,[33,34] whereas other studies showed that IFN-α enhanced the production of IL-4 by PBMCs from patients with chronic hepatitis C.[35] Of note, it has been also demonstrated that IFN-α by itself directly inhibited the production of IL-5 by $CD4^+$ T cells stimulated with PMA and anti-CD28.[19] Although IL-5 production was strongly inhibited in this study, IL-4 production was either upregulated or unchanged by IFN-α.[19] It was thus suggested that the effects of IFN-α on IL-4 production might depend on the system considered. Moreover, it was also possible that the contaminating non-T cells[33-35] or supplemental fibroblasts[19] might result in conflicting results as to the effects of IFN-α on IL-4 expression. In this regard the results in the current studies have clearly demonstrated that IFN-α directly upregulates IL-4 production and mRNA expression in $CD4^+$ T cells stimulated with immobilized anti-CD3 in the complete absence of other cell components, such as NK cells, accessory cells, and B cells.

The time kinetics of IL-5 and IL-13 expression were quite different from those of IL-4 expression in cultures of immobilized anti-CD3–stimulated $CD4^+$ T cells. Thus the production of IL-4 appeared to reach its peak at 24 hours of culture, whereas that of IL-5 and IL-13 markedly increased between 24 and 72 hours, during which the

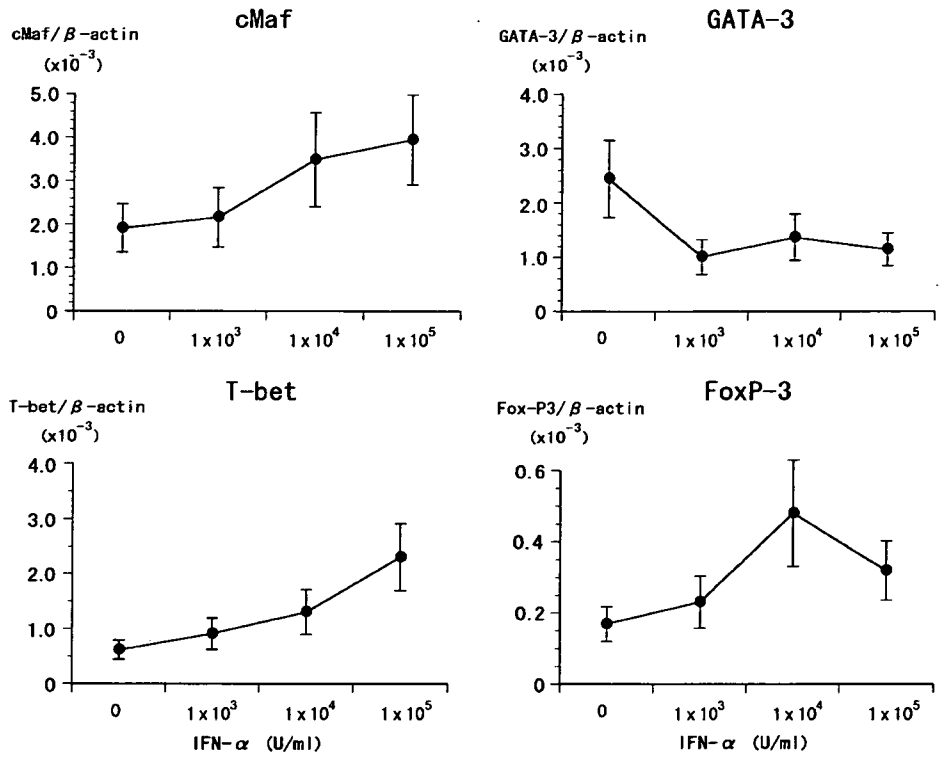

FIG 2. Effects of IFN-α on the expression of mRNA for various transcription factors in anti-CD3–activated $CD4^+$ T cells. $CD4^+$ T cells (2×10^5/well) were cultured in wells with immobilized anti-CD3 (mAb 64.1, 100 ng/well) with various concentrations of IFN-α. After 3 hours of incubation, total RNA was isolated, and real-time quantitative PCR was performed with specific primers for c-Maf, GATA-3, T-bet, Fox-P3, and β-actin. All results were calibrated to the copy number of β-actin (copies per microliter) from each cDNA sample. *Error bars* indicate the SD of duplicated determinations.

production of IL-4 was almost unchanged. Moreover, the upregulation of IL-4 production by IFN-α was clearly observed as early as 24 hours of culture, whereas the downregulation of IL-5/IL-13 production by IFN-α became evident at 72 hours of culture, when the effect of IFN-α on IL-4 production was less marked. These results suggest that IL-4 and IL-5/IL-13 might be expressed at different stages of activation of $CD4^+$ T cells or be expressed in different subsets of $CD4^+$ T cells. In fact, previous studies showed that IL-4 is prominently produced by naive T_H0 cells in contrast to IL-5 and IL-13, which are generally limited to T_H2-like effector-memory cells.[36] Moreover, it has been revealed that the presence of IL-4 in initial priming of $CD4^+$ T cells directs the development of T_H2-like effector cells, although the source of IL-4 initiating this process is debated.[37,38] It is therefore possible that IFN-α might facilitate the development of T_H2 cells through upregulation of IL-4 expression in naive T_H0 cells. Further studies are required to clarify this point.

It has been revealed that the transcription factors c-Maf and GATA-3 are selectively expressed in T_H2 cells and have been shown to regulate T_H2 cytokine expression.[9,10] Thus c-Maf is required for the expression of IL-4,[11] whereas GATA-3 is critical for the expression of IL-5[12] and IL-13.[13] It was therefore possible that the differential effects of IFN-α on the expression of IL-4, IL-5, and IL-13 in anti-CD3–activated $CD4^+$ T cells might result from changes in the expression of these transcription factors. Of note, we demonstrate that IFN-α enhances the expression of c-Maf mRNA, whereas it does not affect the expression of GATA-3 mRNA in $CD4^+$ T cells stimulated with immobilized anti-CD3. These results therefore suggest that IFN-α might enhance the expression of IL-4 in anti-CD3–activated $CD4^+$ T cells by upregulating the expression of c-Maf mRNA. Moreover, it is also suggested that IFN-α might suppress the expression of IL-5 and IL-13 through unknown mechanisms that do not involve the expression of GATA-3 mRNA.

Recent studies have demonstrated the presence of a population of regulatory T cells that developed from naive $CD4^+CD25^-$ T cells during a T_H1 response distinct from $CD25^+$ regulatory T cells.[25] These regulatory T cells expressed Fox-P3 and T-bet and potently inhibited the development of airway hyperreactivity.[25] Of note, we have also shown in the present study that IFN-α upregulates the expression of mRNA for Fox-P3, as well as T-bet, in immobilized anti-CD3–stimulated $CD4^+$ T cells. It is

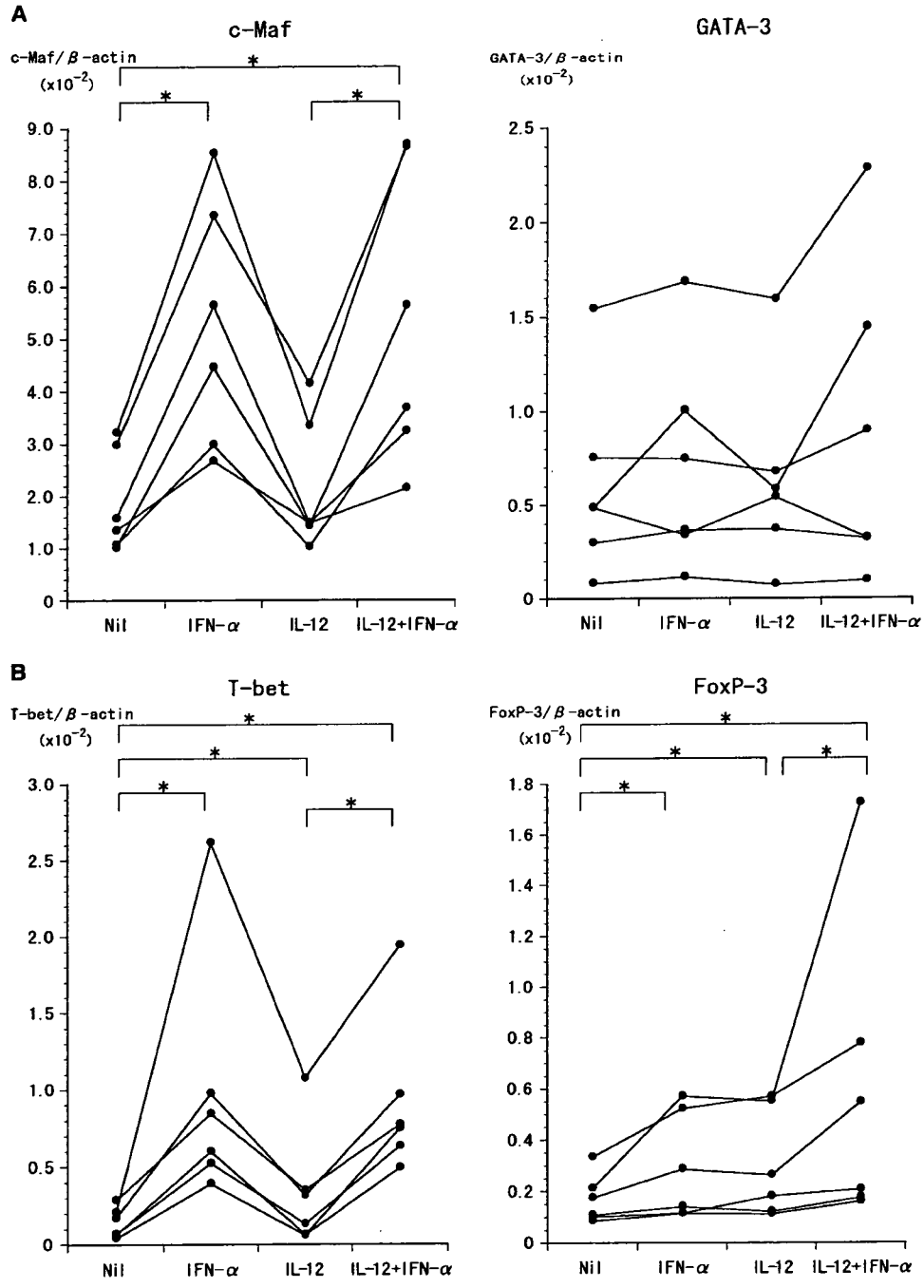

FIG 3. Effects of IFN-α and IL-12 on the expression of mRNA for various transcription factors in anti-CD3–activated CD4$^+$ T cells. CD4$^+$ T cells (2×10^5/well) were cultured in wells with immobilized anti-CD3 (mAb 64.1, 100 ng/well) with or without IFN-α (1×10^5 IU/mL) and IL-12 (10 ng/mL). After 3 hours of incubation, total RNA was isolated, and real-time quantitative PCR was performed with specific primers for c-Maf, GATA-3, T-bet, FoxP3, and β-actin. All results were calibrated to the copy number of β-actin (copies per microliter) from each cDNA sample. Each *line* on the graph is representative of the same cell preparation from the same donor. *$P < .05$.

therefore possible that IFN-α might promote the development of T_H1-like regulatory T cells, which might suppress T_H2 responses, including the expression of IL-5 and IL-13.

In summary, taken together with data from previous studies,[17,19,33] the data in the present study provide a rational basis for therapeutic use of IFN-α therapy in T cell–mediated disorders associated with IL-5 hyperproduction, such as hypereosinophilic syndrome.[39] Of note, it has been recently reported that IFN-α treatment rapidly improved the clinical condition of patients with