Serum Cytokine Levels and Type 1 and Type 2 Intracellular T Cell Cytokine Profiles in Mixed Connective Tissue Disease

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ABSTRACT. ***Objective.*** To determine serum cytokine concentrations and intracellular cytokine production of CD4+ and CD8+ T cells in 20 patients with mixed connective tissue disease (MCTD).

Methods. Detailed analysis of cytokine production; 8 patients were in the active stage, 12 in the inactive stage of the disease. Serum concentrations of interferon-γ (IFN-γ), interleukin 4 (IL-4), and IL-10 were assessed by ELISA. Intracellular content of IFN-γ, IL-4, and IL-10 in CD4+ and CD8+ peripheral blood T cell and lymphocyte subsets was determined by flow cytometry.

Results. Serum concentrations of both type 1 and type 2 cytokines were significantly higher in patients with MCTD than in healthy controls. IFN-γ expression of CD4+ and CD8+ T cells did not differ comparing all patients to controls. In patients with active MCTD, the percentage of CD8+/IFN-γ+ Tc1 cells was significantly increased compared to inactive disease or to controls ($p < 0.05$). IL-4 expression of CD4+ T cells was scarcely detectable in MCTD, while a subpopulation of CD8+ Tc2 cells produced IL-4. A higher percentage of these CD8+/IL-4+ Tc2 cells were detected in patients with MCTD, especially in active disease, compared to controls ($p < 0.05$). The percentage of IL-10-expressing CD4+ and CD8+ T cells was higher in patients than in controls ($p < 0.05$). Again, CD4+ and CD8+ T cells from patients with active MCTD produced significantly more IL-10 than cells in patients with inactive disease or in controls ($p < 0.05$).

Conclusion. Our results suggest that MCTD is associated with increased production of both type 1 (IFN-γ) and type 2 cytokines (IL-4, IL-10). Cytokine production is usually higher in active MCTD than in inactive disease. CD8+ T cells may produce more IFN-γ and IL-10 in comparison to CD4+ T cells. Patients with active disease have more IL-4-expressing Tc2 cells and IL-10-expressing Th2 and Tc2 cells than patients with inactive MCTD or controls. In MCTD, increased IL-10 production by Th2 and Tc2 cells may be an attempt by the immune system to downregulate the inflammatory reaction, although this effect may not be sufficient to restore the physiological Th1/Th2 balance in MCTD. (J Rheumatol 2002;29:2136–42)

Key Indexing Terms:
MIXED CONNECTIVE TISSUE DISEASE
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CD4+ T CELLS
CD8+ T CELLS
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TC1-TC2 CELLS

Mixed connective tissue disease (MCTD) is a systemic autoimmune disease characterized by an array of clinical symptoms associated with the presence of autoantibodies reactive with U small nuclear RNP (U1RNP) autoantigens[1]. The most frequently observed association of symptoms consists of arthritis, Raynaud's phenomenon, myositis, esophageal dysmotility, and acrosclerosis[2,3]. Longterm followup studies reveal that other symptoms, such as serositis (pleuritis, pericarditis), pulmonary involvement (interstitial lung disease, pleural effusions, pulmonary artery hypertension), cutaneous involvement (facial erythema, telangiectasia, hypo-hyperpigmentation), neuropsychiatric disease, and glomerulonephritis, may also develop in MCTD[4-9].

The number and functions of T cells and the array of cytokines produced by T cells and associated mechanisms are less known in MCTD than in other autoimmune diseases such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA). Low numbers of CD8+ T cells have been described in MCTD[10,11], while Alarcon-Segovia, *et al* suggested that anti-RNP autoantibodies may cause the deletion of suppressor T cells[12]. T cells are activated in MCTD,

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and they produce both Th type 1 [interferon-γ (IFN-γ)] and type 2 cytokines [interleukin 10 (IL-10)]. Serum cytokine concentrations were higher in patients with MCTD than patients with SLE or RA[13]. However, we found no reports in the literature regarding intracellular cytokine profiles in MCTD.

We investigated cytokine levels in sera of patients with MCTD compared to healthy controls using ELISA. Patients with MCTD were in the active or inactive stage of the disease, determined by the method of Burdt, *et al*[9]. As serum cytokine concentrations do not indicate the cellular source of cytokines, we used a rapid flow cytometric assay to detect intracellular cytokines in peripheral blood T lymphocytes. Our aim was to determine the distinct cytokine patterns of CD4+/IFN-γ+ Th1 cells, CD4+/IL-4+ and CD4+/IL-10+ Th2 cells, CD8+/IFN-γ+ Tc1 cells, and CD8+/IL-4+ and CD8+/IL-10+ Tc2 cells in both the active and the inactive stage of MCTD and in healthy controls.

MATERIALS AND METHODS

Patients. Twenty patients with MCTD were studied. The diagnosis of MCTD was established according to criteria first described by Alarcon-Segovia and Villareal, which include Raynaud's phenomenon, swelling of the hands with a spindle-like swelling of the fingers, sclerodactyly, polyarthritis, myositis, and the presence of anti-U1RNP autoantibodies in sera. The diagnosis was confirmed by the presence of 4 clinical features in addition to anti-U1RNP seropositivity[14] (Table 1).

Clinically active disease was defined as described by Burdt, *et al*[9]. Briefly, disease was considered inactive when there was no evidence of major organ involvement except mild Raynaud's phenomenon, and patients had normal overall functional activity for more than one year with a regimen of < 20 mg prednisone per day. Patients with actvive disease had severe disease involving major organ systems and required intensive therapy with repeatedly administered prednisone > 30 mg per day (Table 1).

Clinical characteristics of patients with MCTD. As presented in Table 1, 20 patients with MCTD (mean age 51 ± 6 yrs, range 38–63), 19 women and one man, participated in the study. Mean disease duration was 10 ± 5 years (range 5–21). Patients' clinical symptoms are shown in Table 1. All patients fulfilled the criteria for MCTD. Twenty healthy women served as negative controls.

Seven patients received nonsteroidal antiinflammatory drugs (NSAID), while 13 patients were given 5–30 mg per day prednisone. Five patients were treated with 7.5 mg per week methotrexate, one patient with 150 mg per day cyclosporin A, 2 patients with 100 mg per day azathioprine, and 2 patients with 100 mg per day cyclophosphamide.

Patients with MCTD underwent followup visits at the outpatient clinic once every 3 months. Serum samples were collected at every visit during the followup period to detect autoantibodies and samples were stored at -70°C. Heparinized peripheral blood samples for examination by flow cytometry were obtained from patients and controls at the time of the investigation.

Antibodies and reagents. Anti-human IFN-γ-FITC/anti-human IL-4-PE, anti-human CD56-PE, anti-human CD3-FITC/HLA-DR-PE monoclonal antibodies, FACS lysing solution, and FACS permeabilizing solution were purchased from Becton Dickinson (Mountain View, CA, USA). Anti-human CD3-FITC, anti-human CD4-QR, anti-human CD8-FITC, anti-human CD8-QR Mab, brefeldin-A, ionomycin, phorbol-12-myristate-13-acetate (PMA), paraformaldehyde, gentamycin, glutamine, and RPMI-1640 were from Sigma (St. Louis, MO, USA). Anti-human IL-10-PE was obtained from Caltag (Burlingame, CA, USA), while anti-human CD19-PerCP and anti-human CD4-PE were provided by Immunotech (La Brea, CA, USA).

Table 1. Clinical and serological data of patients with MCTD at the time of study assessments. Normal range for anti-U1RNP in our laboratory is < 18 μg/ml. Disease activity was determined by Burdt, *et al*[9].

Patient	Sex	Age, yrs	Duration of Disease, yrs	Active Disease	Organ Involvement	Anti-U1NRP Antibody Levels, μg/ml	Therapy
1	F	52	14	Yes	A, M, I, V	44.1 +	C, MTX, Pf
2	F	47	5	Yes	R, A, S, E	41.3 +	C, MTX, ACE, Pf
3	F	58	16	Yes	R, A, M, E, P, I	23.3 +	C, CYC, ACE, Dic
4	F	49	16	Yes	R, A, M, P, I, V	19.1 +	C, CSA, ACE, Pf
5	F	52	5	Yes	M, E, P, I, V	45.0 +	C, AZA, ACE, Pf, Dic
6	F	58	8	Yes	R, S, E, I	28.8 +	C, AZA, ACE, Pf
7	F	51	6	Yes	R, A, M, E	32.9 +	C, CYC, ACE, Pf
8	F	55	14	Yes	R, A, M	1.2 –	C, MTX, ACE, Pf
9	F	59	12	No	A, S, E	33.1 +	NSAID, Pf
10	F	48	5	No	R, A, V	29.5 +	NSAID, Pf
11	F	38	5	No	R, E, P, V	31.2 +	C, ACE, Pf
12	F	57	6	No	R, M, E	14.5 –	C, MTX, ACE, Pf
13	F	63	17	No	R, A, S	7.9 –	NSAID, ACE, Pf, Dic
14	F	51	21	No	R, A, M, P, I	21.6 +	C, ACE, Pf
15	F	59	17	No	A, E, I, V	1.0 –	NSAID, ACE
16	F	51	15	No	R, M, E	21.4 +	NSAID
17	F	48	8	No	R, A, S, P	16.3 +	NSAID
18	F	39	7	No	R, A, E	5.9 –	C, ACE, Pf
19	M	46	6	No	R, A, E, I	6.8 –	C, MTX, ACE, Pf
20	F	44	9	No	R, M, S, E	24.6 +	NSAID, ACE, Pf

R: Raynaud's phenomenon, A: arthritis, M: myositis, S: sclerodactyly, E: esophageal hypomotility, P: pericarditis or pleuritis, I: interstitial lung disease, V: vasculitis; C: corticosteroid, AZA: azathioprine, CYC: cyclophosphamide, CSA: cyclosporine-A, MTX: methotrexate, ACE: angiotensin convertase inhibitor, Pf: pentoxyfilline, NSAID: nonsteroidal antiinflammatory drugs, Dic: dicoumarol.

Determination of serum cytokine and anti-U1RNP autoantibody levels. We measured the levels of circulating cytokines (IFN-γ, IL-4, IL-10) in the sera of patients and controls using commercial ELISA (OptEIA™ system, Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Autoantibodies to U1RNP were also determined by ELISA (Cogent Diagnostics, Edinburgh, UK). The normal range for this method (seronegativity) was < 18 μg/ml. Anti-U1RNP autoantibody concentrations for patients are shown in Table 1.

Analysis of lymphocyte subsets. Heparinized venous whole blood was stained with anti-human antibodies conjugated by fluorescent dyes for 30 min at room temperature in the dark. (The use of whole blood prevents cell destruction and maintains intercellular interactions in comparison to purified leukocyte subpopulations.) Stained cells were fixed by 2% paraformaldehyde. The fluorescence analysis was performed on an Epics XL-4 flow cytometer (Coulter, Hialeah, FL, USA) with a 488 nm argon laser. Emitted fluorescence was determined by means of 525, 575, and 635 nm band pass filters; 5000 cells were acquired from the list mode and data were analyzed using system II. 3.0. software. Analysis gates were set on lymphocytes according to forward and side scatter properties. Results were expressed as the percentage of surface CD markers in all lymphocytes.

Cell stimulation and staining. Evaluation of intracellular cytokines was according to the protocol described by Maino, *et al*[15], modified by Nagy, *et al*[16]. Briefly, 1 ml whole blood was diluted 1:2 in RPMI-1640 containing 80 mg/l gentamycin and 2 nM glutamine. Cells were stimulated with ionomycin (1 μg/ml), PMA (25 ng/ml), and brefeldin-A (10 μg/ml) for 4 h, as this is the time maximal cytokine expression is usually observed. Unstimulated cells served as controls. Cell stimulation was performed at 37°C in a humified CO_2 incubator. After stimulation, cells were stained in the dark for CD4 and CD8 antigens for 30 min at room temperature. Cell staining was followed by red cell lysis using FACS lysing solution, 10 min at room temperature. Leukocytes were centrifuged (500 g, 10 min) and cell membrane was permeabilized using FACS permeabilizing solution for 10 min at room temperature. Samples were washed with PBS and incubated 30 min in the dark with Mab to IFN-γ, IL-4, and IL-10. Samples were fixed using paraformaldehyde. Samples were measured with a Coulter Epics XL-4 flow cytometer. We determined the rate of IFN-γ, IL-4, and IL-10 positive cells among 5000 CD4+ and CD8+ T cells.

Statistical analysis. Data are given as mean value ± standard deviation. After testing for normality, data were compared by Student t test (paired and unpaired) or Mann-Whitney test and correlated with Spearman's rank correlation. The level of statistical significance used was $p < 0.05$.

RESULTS

Lymphocyte subpopulations in active and inactive MCTD and controls. The percentages of CD3+, CD4+, CD8+, CD19+, CD56+, and CD3+/HLA-DR+ lymphocytes as well as the CD4/CD8 ratio in patients with active and inactive MCTD and healthy controls are listed in Table 2. There was no difference in T cell subsets or the CD4+/CD8+ ratio between patients and controls. The percentages of CD19+ B cells and CD56+ NK cells were also similar comparing patients and controls. However, the expression of HLA-DR, a cell activation marker, was increased on CD3+ T lymphocytes in patients compared to controls (9.2 ± 2.2% vs 1.8 ± 1.4%; $p < 0.05$). There was no difference in the percentage of CD3+, CD4+, CD8+, CD56+, or CD3+/HLA-DR+ T cell subsets when patients with active or inactive MCTD were compared to controls (Table 2).

Analyzing CD19 expression in the active and inactive MCTD subpopulations separately, the percentage of CD19+ cells was significantly decreased in inactive MCTD compared to active disease (5.7 ± 1.9% vs 9.1 ± 5.6%; $p < 0.05$) and to controls (5.7 ± 1.9% vs 11.1 ± 4.1%; $p < 0.05$). Thus, the T cell/B cell ratio was higher in patients with active MCTD. The ratio of CD4+/CD8+ T cells was also significantly elevated in active vs inactive disease (3.1 ± 1.1% vs 2.1 ± 0.6%; $p < 0.05$). The latter difference was due to the higher percentage of CD4+ T cells in the peripheral blood of patients with active in comparison to inactive MCTD. Thus, there may be enrichment of T versus B cells as well as CD4+ versus CD8+ cells in patients with active compared to inactive MCTD.

Anti-U1RNP autoantibody concentrations in active and inactive MCTD. Certainly, all patients with MCTD showed anti-U1RNP seropositivity at the time of diagnosis. At the time of the study, 13/20 patients were seropositive (anti-U1RNP antibody levels > 18 μg/ml). There were 7/8 (87.5%) seropositive patients in the active and 6/12 (50%) seropositive patients in the inactive disease group. Anti-U1RNP antibody concentrations were significantly higher in the active than in the inactive group (30.9 ± 5.3 vs 18.9 ± 3.1 μg/ml, respectively; $p < 0.05$).

Serum cytokine levels in MCTD. Serum concentrations of IFN-γ, IL-4, and IL-10 were significantly higher in patients than controls (IFN-γ 61.8 ± 83.2 vs 20.0 ± 27.8 pg/ml; IL-4 27.5 ± 38.1 vs 5.5 ± 11.0 pg/ml; and IL-10 32.3 ± 33.6 vs 6.9 ± 7.0 pg/ml) ($p < 0.05$) (Table 3). This difference, for all 3 cytokines, was due to patients with active disease.

In active MCTD, the levels of IFN-γ were higher than in

Table 2. T and B cell subsets in the peripheral blood of patients with active and inactive MCTD and healthy controls.

Lymphocyte Subsets	Controls, n = 20, % A	MCTD, n = 20, % B	Active MCTD, n = 8 , % C	Inactive MCTD, n = 12, % D	p, MCTD vs Controls A vs B	p, Active MCTD vs Controls A vs C	p, Inactive MCTD vs Controls A vs D	p, Active vs Inactive MCTD C vs D
CD3+	72.8 ± 5.4	71.5 ± 9.8	75.6 ± 11.2	68.9 ± 15.9	NS	NS	NS	NS
CD56+	8.7 ± 4.1	11.9 ± 7.0	12.6 ± 8.6	10.4 ± 6.2	NS	NS	NS	NS
CD19+	11.1 ± 4.1	9.6 ± 5.5	9.1 ± 5.6	5.7 ± 1.9	NS	NS	< 0.05	< 0.05
CD3+/HLA-DR+	1.8 ± 1.4	9.2 ± 5.5	9.6 ± 6.3	9.1 ± 5.2	< 0.05	< 0.05	< 0.05	NS
CD4+	46.3 ± 6.3	46.6 ± 12.2	51.5 ± 12.4	45.0 ± 11.3	NS	NS	NS	NS
CD8+	18.8 ± 5.7	22.3 ± 10.1	21.1 ± 12.4	21.8 ± 8.8	NS	NS	NS	NS
CD4+/CD8+	2.7 ± 1.0	2.8 ± 2.2	3.1 ± 1.1	2.1 ± 0.6	NS	NS	NS	< 0.05

Table 3. Serum IFN-γ, IL-4, and IL-10 levels in patients with active and inactive MCTD and controls.

Serum Cytokines, pg/ml	Controls, n = 20, % A	MCTD, n = 20, % B	Active MCTD, n = 8, % C	Inactive MCTD, n = 12, % D	p, MCTD vs Controls A vs B	p, Active MCTD vs Controls A vs C	p, Inactive MCTD vs Controls A vs D	p, Active vs Inactive MCTD C vs D
IFN-γ	20.0 ± 27.8	61.8 ± 83.2	74.4 ± 91.7	42.9 ± 69.5	< 0.05	< 0.05	NS	NS
IL-4	5.5 ± 11.0	27.5 ± 38.1	53.2 ± 44.0	10.3 ± 21.8	< 0.05	< 0.05	NS	< 0.05
IL-10	6.9 ± 7.0	32.3 ± 33.6	57.9 ± 41.7	15.2 ± 6.8	< 0.05	< 0.05	NS	< 0.05

inactive disease, but the difference was not statistically significant (74.4 ± 91.7 vs 42.9 ± 69.5 pg/ml). The serum concentrations of IL-4 and IL-10 were significantly increased in active compared to inactive MCTD (IL-4 53.2 ± 44.0 vs 10.3 ± 21.8 pg/ml; IL-10 57.9 ± 41.7 vs 15.2 ± 6.8 pg/ml) ($p < 0.05$) (Table 3). IFN-γ, IL-4, and IL-10 production was also increased in patients with active MCTD compared to controls (IFN-γ 74.4 ± 91.7 vs 20.0 ± 27.8 pg/ml; IL-4 53.2 ± 44.0 vs 5.5 ± 11.0 pg/ml; and IL-10 57.9 ± 41.7 vs 6.9 ± 7.0 pg/ml) ($p < 0.05$) (Table 3). In contrast, cytokine production in inactive MCTD did not differ from controls (Table 3).

Serum cytokine concentrations of patients were also correlated with anti-U1RNP autoantibody concentrations (Tables 1 and 3). However, no correlation was found in anti-U1RNP concentrations between active and inactive stages of disease (data not shown).

Flow cytometric analysis of IFN-γ, IL-4, and IL-10-expressing CD4+ and CD8+ lymphocytes in active and inactive MCTD. IFN-γ expression of CD4+ (Th1 cells) and CD8+ (Tc1 cells) lymphocytes in patients did not differ significantly from controls (CD4+ 20.7 ± 10.4% vs 22.0 ± 6.0%; CD8+ 39.5 ± 17.6% vs 43.4 ± 8.1%) (Table 4). Again, there was no difference in the percentage of CD4+/IFN-γ+ Th1 cells between patients with active and inactive disease (21.4 ± 11.8% vs 20.3 ± 9.9%). Conversely, in active MCTD the percentage of CD8+/IFN-γ+ cells (Tc1 cells) was significantly increased in comparison to patients with inactive disease (55.4 ± 13.0% vs 26.4 ± 7.9%; $p < 0.05$). Moreover, the percentage of these Tc1 cells was also higher in controls compared to patients with inactive MCTD (43.4 ± 8.1% vs 26.4 ± 7.9%; $p < 0.05$) (Table 4). There was no significant difference in the percentage of CD8+/IFN-γ+ Tc1 cells between active MCTD patients and controls (Table 4).

There was a significant decrease of IL-4 expression in CD4+ T cells (CD4+/IL-4+, Th2 cells) derived from patients compared to controls (0.17 ± 0.2% vs 1.1 ± 0.7%; $p < 0.05$). As well, the percentage of IL-4-expressing CD4+ Th2 cells was lower in patients with active MCTD than those with inactive disease (0.1 ± 0.1% vs 0.2 ± 0.3%; $p < 0.05$) or controls (0.1 ± 0.1% vs 1.1 ± 0.7%; $p < 0.05$). On the other hand, we detected a significant increase in the percentage of CD8+/IL-4+ cells (Tc2 cells) in MCTD patients compared to controls (0.8 ± 0.7% vs 0.4 ± 0.2%; $p < 0.05$). The percentage of CD8+/IL-4+ T cells was higher in active than in inactive MCTD (1.5 ± 0.6% vs 0.4 ± 0.2%; $p < 0.05$) or in controls (1.5 ± 0.6% vs 0.4 ± 0.2%; $p < 0.05$) (Table 4).

The percentage of IL-10-producing CD4+ (Th2) and CD8+ (Tc2) cells was increased in patients in comparison to controls (4.5 ± 4.2% vs 2.1 ± 0.6%; $p < 0.05$). In addition, IL-10 production by both CD4+ and CD8+ cells was upregulated in patients with active compared to those with inactive MCTD (8.1 ± 5.3% vs 2.8 ± 1.9% and 7.1 ± 5.7% vs 2.8 ± 1.1%, respectively; $p < 0.05$) and controls (8.1 ± 5.3% vs 2.1 ± 0.6% and 7.1 ± 5.7% vs 1.8 ± 1.0%, respectively; $p < 0.05$) (Table 4).

Anti-U1RNP autoantibody concentrations in MCTD

Table 4. Immunoreactivity of CD4+ and CD8+ T cells producing IFN-γ, IL-4, and IL-10 in patients with active and inactive MCTD and controls.

IFN-γ, IL-4, IL-10 Expressing CD4+ and CD8+T Cell Subsets	Controls, n = 20, % A	MCTD, n = 20, % B	Active MCTD, n = 8, % C	Inactive MCTD, n = 12, % D	p, MCTD vs Controls A vs B	p, Active MCTD vs Controls A vs C	p, Inactive MCTD vs Controls A vs D	p, Active vs Inactive MCTD C vs D
Type 1 cell subsets								
Th1 (CD4+/IFN-γ+)	22.0 ± 6.0	20.7 ± 10.4	21.4 ± 11.8	20.3 ± 9.9	NS	NS	NS	NS
Tc1 (CD8+/IFN-γ+)	43.4 ± 8.1	39.5 ± 17.6	55.4 ± 13.0	26.4 ± 7.9	NS	NS	< 0.05	< 0.05
Type 2 cell subsets								
Th2 (CD4+/IL-4+)	1.1 ± 0.7	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.3	< 0.05	< 0.05	< 0.05	< 0.05
Tc2 (CD8+/IL-4+)	0.4 ± 0.2	0.8 ± 0.7	1.5 ± 0.6	0.4 ± 0.2	< 0.05	< 0.05	NS	< 0.05
Th2 (CD4+/IL-10+)	2.1 ± 0.6	4.5 ± 4.2	8.1 ± 5.3	2.8 ± 1.9	< 0.05	< 0.05	NS	< 0.05
Tc2 (CD8+/IL-10+)	1.8 ± 1.0	4.5 ± 4.1	7.1 ± 5.7	2.8 ± 1.1	< 0.05	< 0.05	NS	< 0.05

patients were also compared with cellular cytokine expression (Tables 1 and 4). However, no correlation was found (data not shown).

DISCUSSION

Type 1 and type 2 T cells, as well as the mediators produced by these cells, play a critical role in the pathogenesis of various autoimmune disorders[17-20]. For example, the "classical" autoimmune disease, SLE, is generally considered to be associated with type 2 T cell and cytokine predominance. However, in lupus nephritis, the balance shows a prominent shift toward a type 1 response[20,21]. Conflicting results have been reported regarding the possible predominance of type 1 or type 2 cytokines in MCTD, but little information is available for this disease compared to autoimmune disorders such as SLE or RA. Some investigators report elevated concentrations of both type 1 and type 2 cytokines in MCTD[13], while others found decreased IL-4 and increased IFN-γ secretion in this condition[22,23]. However, these reports are based on serum cytokine assessments. We were unable to find any literature data on intracellular cytokine production in MCTD.

We assessed serum and intracellular cytokine production in MCTD. Out of 20 patients followed at our outpatient clinic, 8 were in the active and 12 the inactive stage of disease. We determined serum levels and intracellular content of the cytokines IFN-γ, IL-4, and IL-10 in cases of active and inactive MCTD and in healthy controls. Our results indicated that both type 1 (IFN-γ) and type 2 (IL-4, IL-10) cytokine concentrations were significantly higher in the sera of patients with MCTD than in controls. In addition, serum concentrations of all 3 cytokines were elevated in active MCTD in comparison to controls but not in inactive disease. In contrast, serum levels of these 3 cytokines were not different in inactive MCTD compared to controls. Similarly to other investigators[24,25], we detected increased mean levels of anti-U1RNP autoantibodies in the sera of active compared to inactive MCTD patients. Also, the frequency of anti-U1RNP seropositivity was higher in those with active disease. However, no significant correlation was found between serum cytokine levels and the concentrations of anti-U1RNP autoantibodies. Another research group also detected increased levels of IL-10 in the sera of MCTD patients without correlation with disease activity or anti-U1RNP antibody concentrations[25].

The distribution of lymphocyte subsets and the CD4+/CD8+ ratio were similar in the peripheral blood of MCTD patients and controls. We detected higher cell surface HLA-DR expression on T cells in patients in comparison to controls. Comparing active and inactive MCTD, there was enrichment in T over B cells, as well as CD4+ over CD8+ cells. Thus, active MCTD may be associated with the recruitment of T cells, among which CD4+ T cells may be more important in active versus inactive disease.

Regarding intracellular cytokine production, the percentage of IFN-γ-producing CD8+ Tc1 cells was significantly decreased in inactive MCTD compared to patients with active disease. On the other hand, CD4+/IFN-γ+ cells seem to be less important in MCTD.

We observed significantly higher percentages of CD8+/IL-4+ Tc2 cells in MCTD compared to controls. In addition, the frequency of CD8+/IL-4+ Tc2 cells was also higher in active than in inactive MCTD or in controls. In contrast, the percentage of IL-4-producing CD4+ Th2 cells was significantly decreased in patients with active and inactive MCTD compared to controls, and was also somewhat lower in active than in inactive disease. Thus, the percentage of Tc2 cells expressing IL-4 is decreased, while that of Th2 cells expressing the same cytokine is increased, in active MCTD.

The frequency of IL-10-expressing CD4+ as well as CD8+ T cells was significantly increased in MCTD patients in comparison to controls, as well as in patients with active MCTD compared to patients with inactive disease and controls. IL-10 suppresses IFN-γ production by type 1 cells and macrophages. Conversely, IL-10 stimulates the proliferation, differentiation, and antibody production of B cells[17,19]. We found no correlation between the percentage of any T cell subpopulations and anti-U1RNP autoantibody concentrations.

Serum levels of all 3 cytokines were increased in MCTD patients compared to controls, as well as in active MCTD versus controls. The activation of MCTD may be associated with increased T cell/B cell and CD4+/CD8+ T cell ratios. Among T cells, CD8+ cells show upregulated production of both IL-4 and IL-10 in MCTD patients compared to controls, and the intracellular expression of these cytokines is upregulated in active disease. In addition, CD8+ cells also secrete more IFN-γ in active MCTD. These results suggest that among CD8+ cells, IL-4 and IL-10-producing Tc2 cells may play a key role in MCTD, and the frequency of these cells may increase with the activation of the disease. IFN-γ-producing Tc1 cells may be involved mostly in active disease states. In contrast, CD4+ cells produce significantly more IL-10, but less IL-4 in MCTD patients than in controls. In addition, increased IL-10 and suppressed IL-4 production by CD4+ cells was found in active compared to inactive MCTD. IFN-γ-producing CD4+ cells (Th1 cells) do not seem to be important in MCTD. Recently, investigators described a distinct subpopulation of regulatory CD4+ T cells termed Tr1 cells, which synthesize significant amounts of IL-10 but only low levels of IL-4. These particular Th2-like cells depend on IL-10 for their growth, but they are independent of IL-4[26,27]. These data suggest that although both IL-4 and IL-10 are considered type 2 cytokines, Th2 cells producing one cytokine but not the other may have differential regulatory roles in inflammatory diseases such as MCTD.

Our data, together with reports from others, suggest that IL-4 and IL-10-producing CD8+ cells (Tc2 cells), as well as IL-10-producing CD4+ cells (Th2 cells) may be important in the pathogenesis of MCTD. The cells may be highly involved in active in comparison to inactive disease states. IFN-γ-producing CD8+ cells (Tc1 cells) may have additional effects in active MCTD. While the functional role of CD8+ Tc1 cells is well established in a number of conditions, the exact *in vivo* role of CD8+ Tc2 cells remains unclear[28,29]. One possible explanation is that CD8+ Tc2 cells act as suppressor or antiinflammatory cells influencing the production of "helper" cytokines. Tc2 cells can also secrete Th2 cytokines and help B cells in antibody production[30]. There is plenty of evidence for the heterogeneity of CD8+ T cell functions. These cells are able to produce IFN-γ and IL-4[31,32]. An increased number of IL-4-producing CD8+ cells was found in the peripheral blood of patients with systemic sclerosis[33]. In studies of patients with polymyositis (PM) and dermatomyositis (DM), we found no differences regarding intracellular cytokine profiles in PM. On the other hand, there was a decrease in the percentage of CD4+/IFN-γ+ Th1 and CD8+/IFN-γ+ Tc1 subpopulations in patients with active DM. In addition, inactive DM was found to be associated with an increased percentage of CD4+/IL-10+ Th2 cells (unpublished observations).

There seems to be a contradiction regarding serum and intracellular IFN-γ levels in our study. IFN-γ concentrations are increased in patients with MCTD compared to controls, as well as in active versus inactive disease. On the other hand, CD4+ and CD8+ cells do not seem to synthesize more IFN-γ in MCTD than under normal conditions. Evaluation of cytokine concentrations in peripheral blood cells or in the serum may not represent the true picture of an *in situ* inflammatory response, with its particular cytokine and cellular environment. In addition, cytokines are pleiotropic, and they are produced by a number of cell types. It is possible that high concentrations of IFN-γ in the sera of MCTD patients are, apart from T cells, also derived from other cell types. For example, synovial cells may also secrete this cytokine, as described in RA[19].

Regarding the possible role of cytokines in autoantibody production, we suggest that the enhanced production of IFN-γ by CD8+ T cells may trigger inflammatory responses, and IL-4 and IL-10 may stimulate autoantibody production by B cells in MCTD. However, we point out again that we and other investigators have found no direct correlation between serum cytokine and anti-U1RNP autoantibody concentrations[25]. These antibodies must always be present in high concentrations at the time of diagnosis of MCTD. However, patients may be seronegative at any time during the later course of the disease. We have also discussed that seropositivity often does not correlate with clinical activity. Here we found correlations between cytokine production and overall disease activity. Taken together, these data suggest that increased systemic and intracellular cytokine production may be involved in the activation of MCTD, but this may be independent of the production of anti-U1RNP autoantibodies. These cytokines may stimulate the production of autoantibodies other than anti-U1RNP in MCTD. The latter mechanism needs further investigation.

We propose that MCTD is characterized by the elevation of both type 1 and type 2 cytokines in patient sera. Locally, IL-10 may have a great influence on immunoregulation in MCTD, including the Th1/Th2 and Tc1/Tc2 balance. CD8+ T cell derived IL-4 may enhance these effects, and in particular, regulatory CD4+ Th2 cells, possibly Tr1 cells, producing IL-10 but not IL-4 may also be involved in these mechanisms. In MCTD, IL-4 and IL-10 production may lead to the enhancement of Th2 and Tc2 responses, especially in active disease. Restoring the cytokine balance may be beneficial for the outcome of MCTD.

REFERENCES

1. GC Sharp, WS Irvin, EM Tan, RG Gould, HR Holman. Mixed connective tissue disease — an apparently distinct rheumatic disease syndrome associated with a specific antibody to an extractable nuclear antigen. Am J Med 1972;52:148-59.
2. GC Sharp. Diagnostic criteria for classification of MCTD. In: Kasukawa R, Sharp GC, editors. Mixed connective tissue disease and antinuclear antibodies. Amsterdam: Elsevier Science Publishers BV; 1987:23-33.
3. Kasukawa R, Tojo T, Miyawaki S. Preliminary diagnostic criteria for classification of mixed connective tissue disease. In: Kasukawa R, Sharp GC, editors. Mixed connective tissue disease and antinuclear antibodies. Amsterdam: Elsevier Science Publishers BV; 1987:41-7.
4. Szegedi G, Kiss E, Bodolay E, et al. Polysystemic autoimmune diseases — clinical and basic research made in Hungary. Allerg Clin Immunol 1996;8:90-3.
5. Hameenkorpi R, Ruuska P, Forsberg S, Tiilikainen R, Makitalo R, Hakula M. More evidence of distinctive features of mixed connective tissue disease. Scand J Rheumatol 1993;22:63-8.
6. Smolen JS, Steiner G. Mixed connective tissue disease: to be or not to be? Arthritis Rheum 1998;41:768-77.
7. Hoffman RW, Greidinger EL. Mixed connective tissue disease. Curr Opin Rheumatol 2000;12:386-90.
8. Maddison PJ. Mixed connective tissue disease: overlap syndromes. Baillieres Best Pract Res Clin Rheumatol 2000;14:111-24.
9. Burdt MA, Hoffman RW, Deutscher SL, Wang GS, Johnson JC, Sharp GC. Long-term outcome in mixed connective tissue disease. Arthritis Rheum 1999;42:899-909.
10. Melendro EI, Saldate C, Rivero SJ, Alarcon-Segovia D. T-cell subpopulations in the peripheral blood of patients with connective tissue diseases as determined by flow cytometry using monoclonal antibodies. Clin Immunol Immunopathol 1983;27:340-7.
11. Becker H, Langrock A, Federlin K. Imbalance of CD4+ lymphocyte subsets in patients with mixed connective tissue disease. Clin Exp Immunol 1992;88:91-5.
12. Alarcon-Segovia DA, Ruiz-Arguelles A. Suppressor cell loss and dysfunction in mixed connective tissue disease. Arthritis Rheum 1980;23:314-8.
13. Bakri Hassan A, Ronnelid J, Gunnarson I, Karlsson G, Berg L, Lundberg I. Increased serum levels of immunoglobulins, C-reactive protein, type 1 and type 2 cytokines in patients with mixed connective tissue disease. J Autoimmun 1998;11:503-8.

14. Alarcon-Segovia DA, Villareal M. Classification and diagnostic criteria for mixed connective tissue disease. In: Kasukawa R, Sharp GC, editors. Mixed connective tissue disease and antinuclear antibodies. Amsterdam: Elsevier Science Publishers BV; 1987:33-40.
15. Maino VC, Picker LJ. Identification of functional subsets by flow cytometry: intracellular detection of cytokine expression. Cytometry 1998;34:207-15.
16. Nagy G, Pállinger E, Antal-Szalmás P, et al. Measurement of intracellular interferon-gamma and interleukin-4 in whole blood T lymphocytes from patients with systemic lupus erythematosus. Immunol Lett 2000;74:207-10.
17. Prahalad S. Atopy, autoimmunity, and the TH1/TH2 balance. J Pediatr 2000;137:446-9.
18. Berner B, Akca D, Jung T, Muller GA, Reuss-Borst MA. Analysis of Th1 and Th2 cytokines expressing CD4+ and CD8+ T cells in rheumatoid arthritis by flow cytometry. J Rheumatol 2000; 27:1128-35.
19. Szekanecz Z, Koch AE. Cytokines. In: Ruddy S, Harris ED Jr, Sledge CB, editors. Kelley's textbook of rheumatology, 6th ed. Philadelphia: WB Saunders; 2001:275-90.
20. Mitsuteru A, Hitoshi N, Yosuke T, et al. Th1/Th2 balance of peripheral T helper cells in systemic lupus erythematosus. Arthritis Rheum 1999;42:1644-8.
21. Dean GS, Tyrrell-Price J, Crawley E, Isenberg DA. Cytokines and systemic lupus erythematosus. Ann Rheum Dis 2000;59:243-51.
22. Funauchi M, Sugishima H, Minoda M, Horiuchi A. Serum level of interferon-gamma in autoimmune diseases. Tohoku J Exp Med 1991;164:259-67.
23. Funauchi M, Minoda M, Horiuchi A. Relationship between serum interleukin-4 or interferon-gamma level and B cell abnormality in patients with collagen vascular diseases. Ryumachi 1991; 31:493-501.
24. Murata I, Sakamoto Y, Dohi M, et al. Clinical assessment of pulmonary hypertension in collagen diseases. Ryumachi 1989; 29:4-10.
25. Hassan AB, Gunnarsson I, Karlsson G, Klareskog L, Forslid J, Lundberg IE. Longitudinal study of interleukin-10, tumor necrosis factor-a, anti-U1-snRNP antibody levels and disease activity in patients with mixed connective tissue disease. Scand J Rheumatol 2001;30:282-9.
26. Bach JF. Non-Th2 regulatory T cell control of Th1 autoimmunity. Scand J Immunol 2001;54:21-9.
27. Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. J Exp Med 1996;184:19-29.
28. Prasse A, Georges CG, Biller H, et al. Th1 cytokine pattern in sarcoidosis is expressed by bronchoalveolar CD4+ and CD8+ T cells. Clin Exp Immunol 2000;122:241-8.
29. Akdis M, Klunker S, Schliz M, Blaser K, Akdis CK. Expression of cutaneous lymphocyte-associated antigen on human CD4+ and CD8+ Th2 cells. Eur J Immunol 2000;30:3533-41.
30. Kemeny DM, Noble A, Holmes BJ, Diaz Sanches D. Immune regulation: a new role for CD8+ T cells. Immunol Today 1994; 15:107-10.
31. Hamann D, Baars PA, Rep MHG, et al. Phenotypic and functional separation of memory and effector human CD8+ T cells. J Exp Med 1997;186:1407-18.
32. Sandberg JK, Fast NM, Nixon DF. Functional heterogeneity of cytokines and cytolytic effector molecules in human CD8+ T lymphocytes. J Immunol 2001;167:181-7.
33. Tsuji-Yamada J, Nakazawa M, Minami M, Sasaki T. Increased frequency of interleukin 4 producing CD4+ and CD8+ cells in peripheral blood from patients with systemic sclerosis. J Rheumatol 2001;28:1252-8.