INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Flow Cytometric Analysis of Lymphocyte Subpopulations and Th1/Th2 Balance in Patients with Polymyositis and Dermatomyositis

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Abstract

Objective Polymyositis (PM) and dermatomyositis (DM) are idiopathic inflammatory myopathies; autoimmune mechanisms are thought to play an important role in their pathogenesis. We investigated the immunocytochemical characteristics and Th1/Th2 balance of peripheral blood lymphocytes in PM and DM using flow cytometry.

Patients and Methods Eight patients with PM and 13 with DM were enrolled in this study. Of these, 8 patients with DM were examined before and after clinical remission. No patients were receiving any treatment for PM or DM at enrollment. Ten healthy subjects were used as controls.

Results Patients with PM showed significant increases in $CD3^+CD4^+HLA\text{-}DR^+$ ($p<0.01$) and $CD19^+CD23^+$ cells ($p<0.05$), and significant decreases in $CD3^+CD4^+$ ($p<0.005$) and $CD4^+CD45RO^+$ cells ($p<0.05$) compared with controls. Patients with DM showed significant increases in $CD19^+$ ($p<0.05$) and $CD19^+CD23^+$ cells ($p<0.05$), and significant decreases in $CD4^+CD45RO^+$ cells ($p<0.005$) and the $CD4^+CD45RO^+/CD4^+CD45RA^+$ ratio ($p<0.005$) compared with controls. $CD4^+$interferon (IFN)-γ^+ cells and the intracellular IFN-γ/interleukin (IL)-4 ratio in $CD4^+$ cells were significantly lower in patients with DM than in those with PM ($p<0.05$) or controls ($p<0.0005$ and $p<0.001$, respectively). The intracellular IFN-γ/IL-4 ratio in $CD4^+$ cells was significantly increased in DM after clinical remission compared with before ($p<0.05$).

Conclusion Both B and helper T cells are activated in peripheral blood of active PM. Th2 cells predominate in peripheral blood of active DM, and the intracellular IFN-γ/IL-4 ratio in $CD4^+$ cells may be useful as a clinical marker indicating disease activity.

Key words: polymyositis, dermatomyositis, flow cytometry, Th1/Th2 balance, intracytoplasmic cytokine

(Inter Med 47: 1593-1599, 2008)
(DOI: 10.2169/internalmedicine.47.0967)

Introduction

Polymyositis (PM) and dermatomyositis (DM) are idiopathic inflammatory myopathies. Autoimmune mechanisms are thought to play an important role in the pathogenesis of PM and DM, but recent immunohistochemical studies on muscle biopsies have demonstrated some differences in the phenotype of lymphocytes between the two disorders. PM shows a direct attack by $CD8^+$ T cells against muscle fibers expressing the MHC-1-antigen, and cell-mediated cytotoxicity may be the main effector mechanism (1-8), while in DM $CD4^+$ T cells and B cells are seen in the interstitial tissue, mainly in perivascular areas, suggesting predominant involvement of humoral immunity (6-8). DM may be a systemic disorder showing muscle weakness and characteristic skin rash with frequent association of interstitial pneumonia. To clarify whether there are any alterations in peripheral

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Received for publication January 30, 2008; Accepted for publication June 3, 2008
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Table 1. Clinical Profiles of the Patients

Case	Diagnosis	Age/Sex	MMT	CK (IU/L)*		IP	Anti-nuclear antibody
				Before treatment	After treatment		
1	PM	51/F	2	3708		-	-
2	PM	50/M	3	4073		-	-
3	PM	54/F	4	611		+	SS-A
4	PM	28/F	2	13662		-	-
5	PM	77/F	3	7486		-	-
6	PM	68/F	4	1820		+	ENA
7	PM	67/F	4	8229		-	-
8	PM	56/F	4	3935		+	SS-A
9	DM	60/F	3	1653	55	-	-
10	DM	24/M	4	272	106	+	-
11	DM	46/F	4	69	63	+	-
12	DM	58/M	4	5304		-	-
13	DM	65/F	4	696		+	-
14	DM	34/F	4	91	19	+	-
15	DM	53/F	4	189	29	+	-
16	DM	67/F	4	287		-	-
17	DM	83/F	3	248		-	-
18	DM	41/F	4	173	18	+	-
19	DM	58/F	4	255	88	-	-
20	DM	29/F	3	6642	136	-	ENA
21	DM	25/M	4	15480		-	ENA

*Normal range: male 43-272, female 30-165.

MMT : manual muscle testing of proximal muscles, CK : creatine kinase, IP : interstitial pneumonia, PM : polymyositis, DM : dermatomyositis, ENA : extractable nuclear antigen

blood indicative of the pathogenetic mechanism of PM and DM, we investigated subpopulations and cytokine production of lymphocytes using flow cytometry. PM showed significant increases in activated B and helper T (Th) cells compared with controls, while in DM only the former was increased significantly. $CD4^+$interferon (IFN)-γ^+ cells and the intracellular IFN-γ/interleukin (IL)-4 ratio in $CD4^+$ cells were significantly decreased in active DM compared with controls, and both indices increased after clinical remission of disease. Th2 cells were relatively predominant in peripheral blood of active DM, and we postulate that the intracellular IFN-γ/IL-4 ratio in $CD4^+$ cells may be useful as a clinical marker indicating disease activity.

Patients and Methods

Patients

We studied 8 patients with PM (7 women and 1 man; mean age 56±15 years, range 28 to 77; disease duration 7.0±12.0 months) and 13 with DM (10 women and 3 men; mean age 49±18 years, range 24 to 83; disease duration 3.3±2.1 months) who had been consecutively admitted to our department from March 2001 to September 2005. They were diagnosed as having PM (6 definite and 2 probable) or DM (6 definite, 5 probable and 2 possible) according to the criteria proposed by Bohan and Peter (9). Clinical profiles of the patients are summarized in Table 1. All patients were considered to have active disease based on increases in serum creatine kinase (CK) or muscle weakness with or without skin rash. Three patients with DM (cases 10, 11 and 14) were classifiable as the amyopathic subtype with regard to normal levels of serum CK before treatment (10), but they showed clinically definite weakness in proximal muscles. Low titers of the anti-nuclear antibody were found in 3 patients with PM (cases 3, 6 and 8) and 2 with DM (cases 20 and 21), but the anti-Jo-1 antibody was negative in all subjects. Intensive systemic survey showed no abnormal findings suggestive of malignancy in any patients. No patients were receiving any treatment for PM or DM at enrollment.

Ten healthy subjects (7 women and 3 men; mean age 40±14 years, range 25 to 65) were used as controls. The Local Ethics Committee approved this study.

Flow cytometry

Mononuclear cells were separated from 10 mL of heparinized whole blood samples by the Ficoll-Hypaque gradient method in all patients and controls. To investigate phenotypic change of lymphocytes, whole blood samples were taken from 8 patients with DM before and after clinical remission, which was defined as complete recovery of muscle weakness with no increases in CK or skin rash. The interval of sampling between before and after clinical remission was 3 to 29 months (mean 10.9±8.6 months). All 8 patients received oral prednisolone (range 30 to 70 mg/day) for treatment. Because of associated interstitial pneumonia and/or sustained muscle weakness with elevated levels of serum CK, oral cyclophosphamide (50 mg/day) and cyclosporin A (range 175 to 250 mg/day) were additionally used in 1 and 5 patients, respectively. There was no opportunity to obtain whole blood samples after clinical remission in the rest of the DM patients. After being washed twice with cold FACS flow buffer (Becton Dickinson, San Diego, CA, USA), cells were divided into those for detecting surface markers and those for intracytoplasmic staining. To detect surface markers of lymphocytes, cells were resuspended at 5×10^6/mL and an aliquot of 200 μL was put into a 10 mL tube; 20 μL of each appropriate monoclonal antibody (mAb) was then added to these tubes, incubated at 4℃ in the dark for 30 minutes and washed twice with cold FACS flow buffer.

For intracellular staining of cytokines, cells were incubated at 37℃ for 4 hours in 5 mL of RPMI 1640 (Sigma, St. Louis, MO, USA) containing 5% fetal bovine serum (Gibco, Grand Island, NY, USA), 2 mM glutamine (Gibco), 2 μM monensin (GolgiStop, Becton Dickinson), 40 ng/mL of phorbol 12-myristate 13-acetate (Sigma) and 500 ng/mL of ionomycin (Sigma). After being washed twice with cold FACS flow buffer, the cells were incubated with 2% human serum at 4℃ for 30 minutes in order to block Fc receptors. The cells were washed once with cold FACS flow buffer and treated with 250 μL of Cytofix/Cytoperm solution (Becton Dickinson) at 4℃ for 20 minutes. After being washed twice with Perm/Wash solution (Becton Dickinson), cells were resuspended at 5×10^6/mL and an aliquot of 200 μL was put into a 10 mL tube. Twenty μL of each appropriate mAb were then added to these tubes, incubated at 4℃ in the dark for 30 minutes, and washed twice with cold FACS flow buffer.

The labeled cells were analyzed by two- or three-color flow cytometry using FACSCalibur (Becton Dickinson). The gate was set on lymphocytes, and 1×10^4 cells were analyzed to determine the percentages of cells positive for each mAb. The following mAbs were employed in this study: fluorescein isothiocyanate-conjugated mAbs to CD4 (1388.2), CD8 (B9.11), CD16 (3G8) and CD19 (J4.119), phycoerythrin-conjugated mAbs to CD25 (B1.49.9), HLA-DR (88.12.2), CD23 (9P25), CD56 (N901), IL-4 (4D9) and IFN-γ (45.15), phycoerythrin-Texas red-conjugated mAbs to CD45RO (UCHL1) and CD45RA (2H4), and phycoerythrin-cyanin 5.1-conjugated mAb to CD3 (UCHT1). All mAbs were purchased from Immunotech (Marseille, France).

Statistical analysis

To determine statistically significant differences between the controls and PM or DM and between PM and DM, Mann-Whitney's U test was employed for phenotypes and intracellular cytokines of lymphocytes. Simple regression and Wilcoxon's signed rank tests were used for the detection of a significant relationship between serum CK and intracellular cytokines and for comparison between before and after clinical remission in DM, respectively. The results represent the mean±standard deviation where applicable, and a p-level less than 0.05 was considered to be statistically significant. Commercially available statistics software was used for data analysis (StatView for Macintosh, Abacus Concepts, Berkeley, CA, USA).

Results

Subpopulations of peripheral blood lymphocytes

Results of phenotypical analysis of peripheral blood lymphocytes are summarized in Table 2. $CD3^+CD4^+HLA\text{-}DR^+$ ($p<0.01$) and $CD19^+CD23^+$ cells ($p<0.05$) were significantly increased in patients with PM compared with controls. PM patients showed significant decreases in $CD3^+CD4^+$ ($p<0.005$) and $CD4^+CD45RO^+$ cells ($p<0.05$) compared with controls. In patients with DM significant increases were seen in $CD19^+$ ($p<0.05$) and $CD19^+CD23^+$ cells ($p<0.05$) compared with controls, while $CD4^+CD45RO^+$ cells and the $CD4^+CD45RO^+/CD4^+CD45RA^+$ ratio were decreased significantly ($p<0.005$). There were no significant differences in $CD4^+CD25^{high}$ (regulatory T cells) and $CD3^+CD8^+$ cells ($CD8^+$ T cells) between controls and patients with PM or DM. No significant differences were seen in any subpopulations between patients with PM and those with DM, but the $CD4^+CD45RO^+/CD4^+CD45RA^+$ ratio was significantly lower in the latter than in the former ($p<0.05$). No significant differences were seen in any subpopulations of DM between before and after clinical remission.

Intracellular cytokines of peripheral blood lymphocytes

Results of intracytoplasmic cytokines in peripheral blood lymphocytes are demonstrated in Fig. 1. DM patients showed significant decreases in $CD4^+IFN\text{-}\gamma^+$ (Th1) cells ($p<0.0005$) and the intracellular IFN-γ/IL-4 ratio in $CD4^+$ cells (Th1/Th2) ($p<0.001$) compared with controls. These indices showed significantly lower values in patients with DM than in those with PM ($p<0.05$). There were no significant differences in $CD4^+IL\text{-}4^+$ (Th2), $CD8^+IFN\text{-}\gamma^+$ (Tc1) or $CD8^+IL\text{-}4^+$

Table 2. Results of Phenotypical Analyses of Peripheral Blood Lymphocytes

		Controls	Polymyositis	Dermatomyositis
$CD3^+CD4^+$	(%)	43.4	30.1	38.2
	SD	10.9	11.9	16.6
	p value (controls vs. PM or DM)		**0.0045**	0.32
	p value (PM vs. DM)			0.25
$CD4^+CD25^{high}$	(%)	0.84	1.17	1.26
	SD	0.33	0.80	0.85
	p value (controls vs. PM or DM)		0.53	0.26
	p value (PM vs. DM)			0.59
$CD3^+CD4^+CD25^+$	(%)	4.42	4.23	4.89
	SD	0.64	2.17	2.62
	p value (controls vs. PM or DM)		0.92	>0.99
	p value (PM vs. DM)			0.82
$CD3^+CD4^+HLA\text{-}DR^+$	(%)	1.13	2.44	1.84
	SD	0.39	1.60	1.83
	p value (controls vs. PM or DM)		**0.0087**	0.41
	p value (PM vs. DM)			0.099
$CD4^+CD45RO^+$	(%)	25.2	18.5	13.5
	SD	6.19	9.55	7.07
	p value (controls vs. PM or DM)		**0.021**	**0.0024**
	p value (PM vs. DM)			0.16
$CD4^+CD45RA^+$	(%)	25.3	17.9	29.9
	SD	12.5	6.42	14.2
	p value (controls vs. PM or DM)		0.16	0.47
	p value (PM vs. DM)			0.090
$CD4^+CD45RO^+$/ $CD4^+CD45RA^+$ ratio		1.26	1.05	0.53
	SD	0.71	0.34	0.31
	p value (controls vs. PM or DM)		0.86	**0.0045**
	p value (PM vs. DM)			**0.0043**
$CD3^+CD8^+$	(%)	20.6	22.5	18.4
	SD	5.3	9.05	11.9
	p value (controls vs. PM or DM)		0.7898	0.2914
	p value (PM vs. DM)			0.2801
$CD19^+$	(%)	13.8	25.7	24.9
	SD	5.20	13.7	12.3
	p value (controls vs. PM or DM)		0.059	**0.021**
	p value (PM vs. DM)			0.76
$CD19^+CD23^+$	(%)	5.58	13.7	11.3
	SD	3.29	8.16	5.55
	p value (controls vs. PM or DM)		**0.027**	**0.025**
	p value (PM vs. DM)			0.76
$CD3^+CD16/CD56^+$	(%)	4.02	8.03	4.25
	SD	1.38	6.08	3.05
	p value (controls vs. PM or DM)		0.18	0.95
	p value (PM vs. DM)			0.076
$CD3^-CD16/CD56^+$	(%)	17.3	18.6	14.7
	SD	10.1	5.84	12.4
	p value (controls vs. PM or DM)		0.48	0.29
	p value (PM vs. DM)			0.16

PM: polymyositis, DM: dermatomyositis, SD: standard deviation

(Tc2) cells between patients with DM and those with PM or controls (Fig. 1A). $CD4^+IFN\text{-}\gamma^+$ cells and the intracellular IFN-γ/IL-4 ratio in $CD4^+$ cells were increased in almost all DM patients examined after clinical remission compared with before, and significant differences were seen between them ($p<0.05$) (Fig. 2). There were no significant differences in any intracellular cytokines between patients with PM and controls. Both PM and DM patients showed no significant relationship between serum CK and any intracellular cytokines. No significant differences were seen in any intracellular cytokines between with and without associated interstitial pneumonia.

Discussion

B cells in peripheral blood have been reported to show a significant increase in DM compared with healthy subjects or PM (11-13). The present study showed $CD19^+CD23^+$ cells to be significantly higher in both DM and PM than in healthy subjects. Considering that CD23 is a surface marker appearing in the earliest phase of B cell activation (14), B cells may be activated in DM and also in PM. T cells in pe-

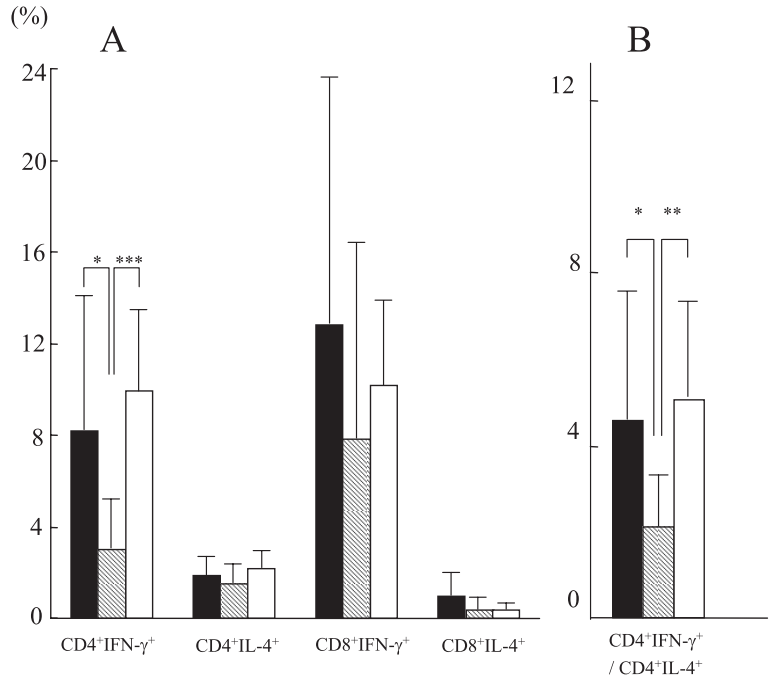

Figure 1. **Analyses of intracellular cytokines of peripheral blood lymphocytes in patients with polymyositis (PM) and dermatomyositis (DM). Results show significant decreases in $CD4^+IFN\text{-}\gamma^+$ cells (A, $p<0.0005$) and the $CD4^+IFN\text{-}\gamma^+/CD4^+IL\text{-}4^+$ ratio (B, $p<0.001$) in DM patients compared with controls. There were significant differences in both indices even between patients with DM and those with PM ($p<0.05$). Closed columns: patients with PM, shaded columns: patients with DM, open columns: controls. IFN-γ: interferon-gamma, IL-4: interleukin-4, *$p<0.05$, **$p<0.001$, ***$p<0.0005$.**

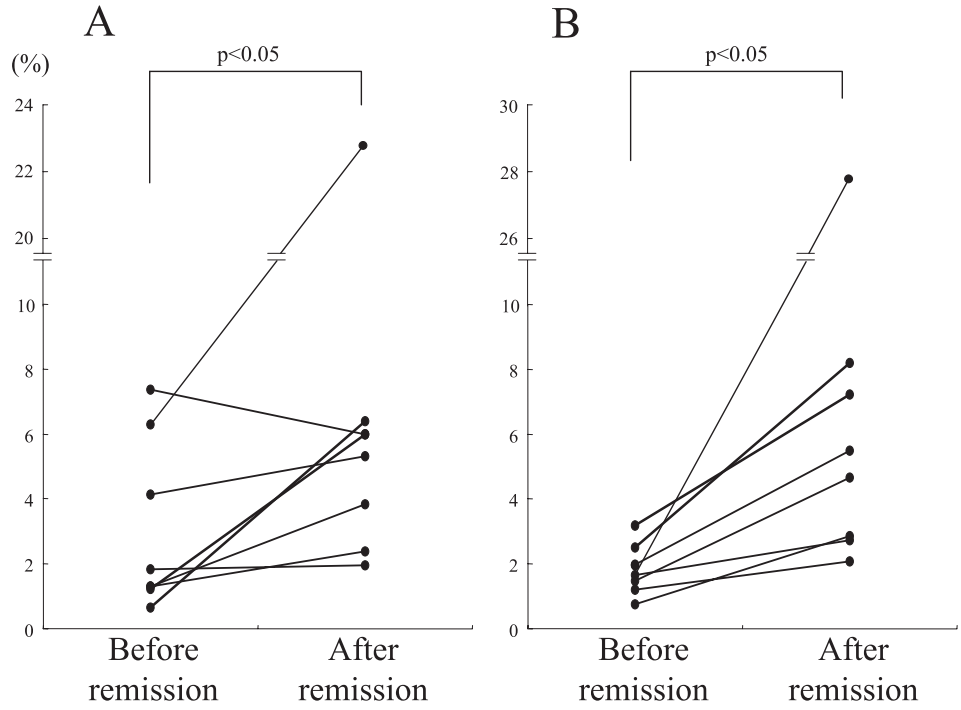

Figure 2. **Intracellular cytokines of peripheral blood lymphocytes in patients with dermatomyositis (DM) before and after clinical remission. DM patients show significant increases in $CD4^+IFN\text{-}\gamma^+$ cells (A, $p<0.05$) and the $CD4^+IFN\text{-}\gamma^+/CD4^+IL\text{-}4^+$ ratio (B, $p<0.05$) after clinical remission compared with before. IFN-γ: interferon-gamma, IL-4: interleukin-4.**

ripheral blood of DM and PM still remain controversial largely because their subpopulations strongly depend on disease phase and activity at sampling (11, 12, 15-17). In our study, flow cytometry was performed in the active phase of

DM and PM, and the latter showed a significant increase and decrease in $CD3^+CD4^+HLA\text{-}DR^+$ cells and $CD3^+CD4^+$ cells compared with healthy subjects, respectively. These results suggest that activated helper T cells may be increased in peripheral blood of active PM. Several histopathological studies have demonstrated perivascular infiltration of B cells and $CD4^+$ T cells in muscles of PM patients (6, 7), and our results support the hypothesis that both cells also play an important role in the pathogenesis of this disorder in addition to $CD8^+$ T cells. $CD4^+$ T cells expressing large amounts of CD25 are considered to have regulatory functions for the immune response (18), but our study showed no significant differences in this subpopulation between healthy subjects and PM or DM.

DM patients showed significant decreases in $CD4^+CD45RO^+$ cells and the $CD4^+CD45RO^+/CD4^+CD45RA^+$ ratio compared with healthy subjects in our study. These results suggest that memory T cells may be decreased in peripheral blood of active DM. Nevertheless, predominant infiltration of $CD4^+CD45RO^+$ cells has been reported to exist in muscles of DM on immunohistochemistry (19). This discrepancy between peripheral blood and muscle tissues can be explained by the highly migratory function of memory T cells. Several reports have suggested that the primary antigenic target in DM is the endothelium of capillaries (10, 20). $CD4^+CD45RA^+$ cells may differentiate into $CD4^+CD45RO^+$ cells quickly after contact with endothelia in muscles, resulting in easy migration of the latter from peripheral blood to the interstitial tissue in active DM (21). In our study PM patients also showed a significant decrease in $CD4^+CD45RO^+$ cells compared with healthy subjects. Considering that there was no significant difference in the $CD4^+CD45RO^+/CD4^+CD45RA^+$ ratio between PM patients and healthy subjects, however, the decrease in $CD4^+CD45RO^+$ cells may be less meaningful in PM than in DM.

$CD4^+$ T cells are classified into Th1 and Th2 according to the production of cytokines, such as IFN-γ and IL-4 respectively. Several reports have suggested that the Th1/Th2 balance may not only contribute to disease activity but also to the pathogenesis of some autoimmune disorders, including systemic lupus erythematosus and myositis (11, 22-24). The present study of intracytoplasmic cytokines using flow cytometry showed that $CD4^+IFN\text{-}\gamma^+$ cells and the $CD4^+IFN\text{-}\gamma^+/CD4^+IL\text{-}4^+$ ratio were significantly decreased in DM compared with healthy subjects or PM. Both of the above-mentioned indices increased after clinical remission of DM in almost all patients examined. These results suggest that Th2 cells may predominate in the peripheral blood of active DM and that the Th1/Th2 ratio is useful as a clinical marker indicating disease activity. A recent report also has demonstrated that the Th1/Th2 ratio in peripheral blood is significantly lower in active DM than in healthy subjects, although the treatment that each patient was receiving at sampling and its influence on intracytoplasmic cytokines are unclear (11). In the present study no patient with DM was receiving any treatment at sampling before clinical remission. All patients with DM were thought to clinically belong to the active phase of disease, and Th2-predominancy is ascribable to DM itself. PM patients showed no significant change in the Th1/Th2 balance compared with healthy subjects.

In conclusion, flow cytometric analysis of peripheral blood lymphocytes revealed activation of B cells and helper T cells in PM and a decrease in the Th1/Th2 ratio in DM. Th2-predominancy in DM may contribute to the production of the pathognomonic autoantibody with regard to activation of B cells, and the Th1/Th2 ratio increases in conjunction with recovery from disease. To further investigate whether the Th1/Th2 balance is involved in the pathogenesis of DM and PM, immunohistochemical studies of lymphocytes infiltrating muscle tissues are required.

Acknowledgement

This work was supported by a grant from Neuroimmunological Disease Division, the Ministry of Public Health, Labor and Welfare, Japan.

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