Clinical and Experimental Rheumatology 2008; 26: 1079-1082.

Analysis of peripheral blood lymphocytes using flow cytometry in polymyalgia rheumatica, RS3PE and early rheumatoid arthritis

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

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Received on January 2, 2008; accepted in revised form on April 14, 2008.

Key words: Polymyalgia rheumatica, remitting seronegative symmetrical synovitis with pitting edema, early rheumatoid arthritis, flow cytometry, intracellular cytokine.

Competing interests: none declared.

ABSTRACT

Objective. *Clinical pictures of polymyalgia rheumatica (PMR) and remitting seronegative symmetrical synovitis with pitting edema (RS3PE) are often indistinguishable from those of early rheumatoid arthritis (RA). To investigate whether there is a difference in immunological aspects among these 3 disorders, we performed a phenotypic analysis of peripheral blood lymphocytes.*

Patients and methods. *Eleven patients with early RA, 14 with PMR and 11 with RS3PE were enrolled in this study. After separation of mononuclear cells from peripheral blood using the Ficoll-Hypaque method, surface markers and intracellular cytokines of lymphocytes were analyzed by 2- or 3-color flow cytometry.*

Results. *Both PMR and RS3PE showed a significant decrease in $CD8^+CD25^+$ cells ($p<0.05$), and significant increases in $CD4^+IFN\text{-}\gamma^+IL\text{-}4^-$ ($p<0.05$), $CD8^+IFN\text{-}\gamma^+IL\text{-}4^-$ ($p<0.05$ and $p<0.01$, respectively) and $CD4^+TNF\text{-}\alpha^+$ cells ($p<0.05$) compared with early RA. $CD3^+CD4^+$ cells were higher in PMR than in RS3PE ($p<0.01$), but there were no significant differences in any other phenotypes between these disorders.*

Conclusions. *A decrease in activated cytotoxic/suppressor T cells and increases in circulating Th1 and Tc1 cells may be common characteristics of PMR and RS3PE in comparison with early RA. Both disorders are clearly different from early RA, and probably belong to the same disease entity with regard to phenotypes of peripheral blood lymphocytes.*

Introduction

Polymyalgia rheumatica (PMR) is an inflammatory disease characterized by pain and stiffness in the shoulder and pelvic girdle, which preferentially affects the elderly (1-3). According to recent radiographical studies extraarticular synovial tissues, such as bursae and tendon sheaths, may be the primary site of inflammation in PMR (4, 5). This disease responds well to low-dose oral prednisolone, but sometimes produces serious impairment in daily living if left untreated (3). Rheumatoid arthritis (RA) also shows pain and stiffness around joints ascribable to synovitis in addition to polyarthralgia, and it is often difficult to clinically discriminate PMR from RA, particularly in the early phase of disease (6, 7). Several reports have demonstrated that 4.8-27% of PMR patients are reclassified as RA in the follow-up survey (6-8). Another inflammatory disease in the synovial tissue is remitting seronegative symmetrical synovitis with pitting edema (RS3PE), which is characterized by remarkable swelling of both hands and feet, sometimes with polyarthralgia (9-11). Based on clinical findings that the edema seen in RS3PE can develop also in PMR (12-14), both disorders have recently come to be considered as different phenotypes of a single clinical entity (10).

Several reports have demonstrated that helper T cells and cytokines secreted from them may play an important role in the pathogenesis of PMR, early RA and RS3PE, although the precise mechanisms remain unclear (15-19). To investigate whether there is a difference in immunological aspects among these 3 disorders, we performed a phenotypic analysis of peripheral blood lymphocytes (PBL) using flow cytometry. Both PMR and RS3PE showed a significant decrease in $CD8^+CD25^+$ cells and significant increases in IFN-γ-producing $CD4^+$ and $CD8^+$ cells compared with early RA. There were no significant differences between PMR and RS3PE except for $CD3^+CD4^+$. We postulate that PMR and RS3PE are clearly different from early RA, and may belong to the same disease entity with regard to surface markers and intracellular cytokines of PBL.

Patients and methods

Patients

We studied 11 Japanese patients with RA (6 women and 5 men; age range, 54-77 years; mean age, 60.1±6.4 years; mean disease duration, 2.5±0.7 months), 14 with PMR (4 women and 10 men; age range, 56-81 years; mean age, 65.5±8.1 years; mean disease duration, 2.4±0.9 months) and 11 with RS3PE (7 women and 4 men; age range, 56-82 years; mean age, 68.0±8.5 years; mean disease duration, 2.5±0.9 months). All the

patients were diagnosed as having RA, PMR or RS3PE in our hospital according to classification criteria established for each disease (1, 10, 20). We excluded patients who fulfilled two or more of the classification criteria for these 3 disorders, in order to highlight immunological differences. The diagnoses of all the patients were unchanged in the follow-up observation for 1 year after this study. No patients were receiving either corticosteroid or disease-modifying anti-rheumatic drugs at sampling. Age-matched healthy subjects (4 women and 3 men; age range, 60-74 years; mean age, 66.6±5.8 years) were employed as a control. The Local Ethics Committee approved this study, and we obtained informed written consent from each patient.

Flow cytometry

Ten ml of heparinized whole blood samples were taken from the patients, and mononuclear cells were separated by the Ficoll-Hypaque gradient method. All the patients were in the active phase of illness within 4 months after onset at sampling. 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 $5x10^6$/ml and an aliquot of 200µl was put into a 10 ml tube. Twenty µl of each appropriate monoclonal antibody (mAb) were then added to these tubes, incubated at 4°C in the dark for 30 min and washed twice with cold FACS flow buffer. The following mAbs were employed in this study: fluorescein isothiocyanate-conjugated (FITC) mAbs to CD4 (13B8.2), CD8 (B9.11), and CD23 (9P25), phycoerythrin-conjugated (PE) mAbs to CD25 (B1.49.9), HLA-DR (B8.12.1), CD80 (MAB104), and CD86 (HA5.2B7), and phycoerythrin-cyanin 5.1-conjugated mAb to CD3 (UCHT1) and CD19 (J4.119). All mAbs were purchased from Immunotech (Marseille, France).

For intracellular staining of cytokines, cells were incubated at 37°C for 4 hrs in 5 ml of RPMI 1640 (Sigma, St. Louis, MO, USA) containing 5% fetal bovine serum (Gibco, Grand Island, NY, USA), 2mM glutamine (Gibco), 2µM 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°C for 30 min 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°C for 20 min. After being washed twice with Perm/Wash solution (Beckton Dickinson), cells were resuspended at $5x10^6$/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°C in the dark for 30 min and washed twice with cold FACS flow buffer. The following mAbs were used: FITC-conjugated mAbs to CD4 and CD8, and PE-conjugated mAbs to interleukin (IL)-4 (4D9, Immunotech), interferon (IFN)-γ (45.15, Immunotech) and tumor necrosis factor (TNF)-α (188, Immunotech).

The labeled cells were analyzed by two- or three-color flow cytometry using FACSCalibur (Becton Dickinson). The gate was set on lymphocytes, and $1x10^4$ cells were analyzed to determine the percentages of cells positive for each mAb.

Statistical analysis

To determine statistically significant differences among PMR, early RA and RS3PE, Mann-Whitney U-test and the Kruskal-Wallis test were employed for phenotypes of PBL and clinical profiles of the patients, respectively. The correlation coefficient test was used for detection of a significant relationship between inflammatory reactions and surface markers or intracellular cytokines of PBL. 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

C-reactive protein (CRP) in serum and the erythrocyte sedimentation rate were 2.28±3.43 mg/dl and 53.5±33.1 mm/hr in RA, 5.66±3.46 mg/dl and 49.9±29.3 mm/hr in PMR, and 7.29±4.52 mg/dl and 49.3±46.1 mm/hr in RS3PE, respectively. There was no significant difference among these 3 disorders. Rheumatoid factor was positive in 8 patients with RA and 1 with PMR. Anti-nuclear antibody could be detected in 1 patient with RA, but the titer was lower than x320 and there were no clinical symptoms suggestive of other associated collagen diseases. The classical 28-joint disease activity score (DAS28) in RA patients was 4.59±0.53 (range, 3.69-5.34) (21). No patients with PMR showed any cranial symptoms suggestive of associated giant cell arteritis. Neither malignancy nor infection could be found in any patients by intensive systemic survey.

The results of flow cytometry are summarized in Table I. $CD3^+CD4^+HLA\text{-}DR^+$ cells were significantly higher in RA ($p<0.05$), PMR ($p<0.05$) and RS3PE ($p<0.01$) than in controls. PMR and RS3PE showed lower levels of $CD3^+CD8^+$ and $CD8^+CD25^+$ cells compared with controls, but there was no significant difference between controls and the two diseases. Conversely, RA showed a slightly higher level of $CD8^+CD25^+$ cells compared with controls, and significant difference was seen in this subpopulation between RA and PMR or RS3PE ($p<0.05$). There was no significant difference in any other surface markers of PBL between controls and RA, PMR or RS3PE. $CD3^+CD4^+$ cells were significantly increased in PMR in comparison with RS3PE ($p<0.01$), but other surface markers showed no significant difference between the two diseases.

In intracytoplasmic cytokines, $CD4^+IFN\text{-}\gamma^+IL\text{-}4^-$, $CD8^+IFN\text{-}\gamma^+IL\text{-}4^-$ and $CD4^+TNF\text{-}\alpha^+$ cells were higher in PMR and RS3PE than in controls, although there was no significant difference between controls and the two diseases. Conversely, RA showed decreases in these subpopulations in comparison with controls. PMR and RS3PE showed significant increases in $CD4^+IFN\text{-}\gamma^+IL\text{-}4^-$ ($p<0.05$), $CD8^+IFN\text{-}\gamma^+IL\text{-}4^-$ ($p<0.05$ and $p<0.01$, respectively) and $CD4^+TNF\text{-}\alpha^+$ cells ($p<0.05$) compared with RA. $CD8^+IFN\text{-}\gamma^-IL\text{-}4^+$ cells were

Table I. Results of phenotypical analysis of lymphocytes using flow cytometry.

		Control	RA	PMR	RS3PE
$CD3^+CD4^+$ (%)		35.3	32.2	42.5	30.0
	SD	7.8	16.6	9.6	11.4
	p-value (*vs.* control)		0.75	0.062	0.37
	p-value (*vs.* RA)			0.14	0.82
	p-value (*vs.* PMR)				**0.009**
$CD3^+CD8^+$ (%)		17.8	17.5	13.4	12.6
	SD	2.2	7.1	5.9	8.2
	p-value (*vs.* control)		0.90	0.24	0.12
	p-value (*vs.* RA)			0.16	0.11
	p-value (*vs.* PMR)				0.55
$CD4^+CD25^+$ (%)		5.5	6.3	7.0	6.4
	SD	2.1	4.7	3.0	3.3
	p-value (*vs.* control)		0.82	0.33	0.59
	p-value (*vs.* RA)			0.55	0.62
	p-value (*vs.* PMR)				0.70
$CD8^+CD25^+$ (%)		0.96	1.12	0.69	0.42
	SD	0.53	0.81	0.86	0.33
	p-value (*vs.* control)		0.90	0.12	0.058
	p-value (*vs.* RA)			**0.046**	**0.020**
	p-value (*vs.* PMR)				0.57
$CD3^+CD4^+$ HLD-DR^+ (%)	0.86	2.54	2.59	3.55	
	SD	0.41	1.46	1.66	2.54
	p-value (*vs.* control)		**0.011**	**0.018**	**0.007**
	p-value (*vs.* RA)			0.94	0.38
	p-value (*vs.* PMR)				0.35
$CD19^+CD86^+$ (%)		0.11	0.52	0.13	0.21
	SD	0.07	0.95	0.19	0.18
	p-value (*vs.* control)		0.50	0.43	0.26
	p-value (*vs.* RA)			0.18	0.87
	p-value (*vs.* PMR)				0.12
$CD19^+CD80^+$ (%)		1.04	0.62	0.71	0.94
	SD	0.70	0.79	0.30	0.91
	p-value (*vs.* control)		0.057	0.43	0.42
	p-value (*vs.* RA)			0.12	0.26
	p-value (*vs.* PMR)				0.91
$CD19^+CD23^+$ (%)		17.5	9.1	8.9	16.1
	SD	10.9	6.2	5.3	12.6
	p-value (*vs.* control)		0.094	0.12	>0.999
	p-value (*vs.* RA)			>0.999	0.15
	p-value (*vs.* PMR)				0.14
CD4+IFN-γ^+ IL-4^- (%)		2.73	2.09	5.53	5.67
	SD	1.80	3.32	4.40	3.97
	p-value (*vs.* control)		0.062	0.38	0.14
	p-value (*vs.* RA)			**0.019**	**0.042**
	p-value (*vs.* PMR)				0.78
$CD4^+$IFN-γ^- IL-4^+ (%)		1.08	1.16	0.90	1.01
	SD	0.59	1.36	0.65	0.65
	p-value (*vs.* control)		0.34	0.55	0.79
	p-value (*vs.* RA)			0.70	0.65
	p-value (*vs.* PMR)				0.53
$CD8^+$IFN-γ^+ IL-4^- (%)		5.55	2.50	6.61	7.49
	SD	2.88	4.66	4.76	5.05
	p-value (*vs.* control)		0.051	0.90	0.47
	p-value (*vs.* RA)			**0.031**	**0.006**
	p-value (*vs.* PMR)				0.62
$CD8^+$IFN-γ^- IL-4^+ (%)		1.20	0.89	0.38	0.24
	SD	1.49	1.17	0.43	0.28
	p-value (*vs.* control)		0.44	0.19	**0.041**
	p-value (*vs.* RA)			0.46	0.38
	p-value (*vs.* PMR)				0.48
$CD4^+$TNF-α^+ (%)		22.9	11.3	26.0	25.9
	SD	4.23	16.5	12.7	15.0
	p-value (*vs.* control)		0.068	0.52	0.65
	p-value (*vs.* RA)			**0.019**	**0.039**
	p-value (*vs.* PMR)				0.78

Bold values are significant at $p<0.05$.
IFN: interferon; IL: interleukin; TNF: tumor necrosis factor; SD: standard deviation.

significantly lower in RS3PE than in controls ($p<0.05$). There was no significant difference in any intracytoplasmic cytokines between PMR and RS3PE. No significant correlation was seen between inflammatory reactions, as shown by CRP and ESR, and surface markers or intracytoplasmic cytokines of PBL (data not shown).

Discussion

To compare subpopulations of PBL among different disorders, age- and gender-matched patients should basically be enrolled in the study. In this respect the PMR patients in our study were male-dominant, and slightly different from other groups, including controls. According to a recent report, however, PBL subpopulations, such as $CD4^+$ and $CD8^+$, show no significant differences between males and females in elderly people as used in our study (22). For surface markers of PBL, we obtained significantly lower levels of $CD8^+CD25^+$ cells in PMR than in early RA. This subpopulation indicates activated cytotoxic/suppressor T cells. $CD3^+CD8^+$ cells (total cytotoxic/suppressor T cells) have been shown to decrease in the active phase of PMR compared with healthy controls (23-25), but in our study there was no significant difference in this subpopulation between early RA and PMR. It remains controversial whether PMR shows significantly lower levels of total cytotoxic/suppressor T cells compared with RA (7, 8). $CD8^+CD25^+$ cells might be more useful than $CD3^+CD8^+$ cells in discriminating PMR from early RA. No significant differences could be detected in either activated helper/inducer T ($CD3^+CD4^+$HLA-DR^+) or B cells ($CD19^+CD86^+$, $CD19^+CD80^+$ and $CD19^+CD23^+$) between PMR and early RA, although the former showed significantly higher levels in both disorders than in controls.

Intracytoplasmic cytokines revealed higher levels of $CD4^+$IFN-γ^+IL-4^- (type 1 helper/inducer T cells, Th1) and $CD8^+$IFN-γ^+IL-4^- (type 1 cytotoxic/suppressor T cells, Tc1) cells in PMR than in RA. Several reports have demonstrated that in RA Th1 cells are predominantly seen in the synovial tissue and fluid but not in peripheral blood (17, 26-28). There are two clinical characteristics supporting a possible increase in IFN-γ-producing T cells in peripheral blood of PMR. One is extended involvement of extraarticular synovial tissues from the early phase of illness. Pain and stiffness in PMR usually develop in a relatively large portion of proximal muscles, including the shoulder and pelvic girdle, while in early RA synovial inflammation is often limited to a few joints. Th1 and Tc1 cells may more easily migrate through peripheral blood and act as the pathognomonic cells in PMR than in RA. The other clinical characteristic is frequent vascular involvement. An immunohistopathological study has demonstrated that IFN-γ can be detected in the temporal artery of

giant cell arteritis, which is sometimes associated with PMR (15). Considering that vascular involvement similar to giant cell arteritis has been shown to exist also in the synovial tissue of PMR (25, 29), Th1 and Tc1 cells may be related to the pathogenesis of PMR with regard to producing IFN-γ. $CD4^+TNF\text{-}\alpha^+$ cells were significantly higher in PMR than in early RA in our study, and this result also suggests that activation of T cells may occur more markedly in peripheral blood of the former than the latter (30).

RS3PE showed a significant decrease in $CD8^+CD25^+$ cells and significant increases in $CD4^+IFN\text{-}\gamma^+IL\text{-}4^-$, $CD8^+IFN\text{-}\gamma^+IL\text{-}4^-$ and $CD4^+TNF\text{-}\alpha^+$ cells compared with early RA as seen in PMR. There were no significant differences in surface markers and intracytoplasmic cytokines between PMR and RS3PE except for $CD3^+CD4^+$. These results suggest that PMR and RS3PE may be different from RA, and have a common immunological background. Similarities in synovitis demonstrated by magnetic resonance imaging and ^{67}Ga-scintigram between PMR and RS3PE also support the hypothesis that both disorders are closely related (31).

In conclusion, both PMR and RS3PE showed a significant increase in IFN-γ-producing T cells and a significant decrease in activated cytotoxic/suppressor T cells compared with early RA. PMR and RS3PE may belong to a single clinical entity with regard to phenotypes of PBL, while RA is clearly different from both disorders. Flow cytometry might be able to contribute to discriminating PMR and RS3PE from early RA, particularly when the cause of synovitis or polyarthralgia is unidentifiable even with routine laboratory examinations.

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