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Alterations in Levels of $CD28^-/CD8^+$ Suppressor Cell Precursor and $CD45RO^+/CD4^+$ Memory T Lymphocytes in the Peripheral Blood of Multiple Sclerosis Patients

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A comprehensive peripheral blood immunophenotype analysis of 16 multiple sclerosis (MS) patients was performed by three-color flow cytometric analysis, and the results were compared with those for age-matched healthy controls. The cell subsets quantified included T cells ($CD3^+$), B cells ($CD19^+$), NK cells ($CD56^+$), $CD4^+$ and $CD8^+$ T cells, cytotoxic ($CD28^+$) and suppressor precursor ($CD28^-$) $CD8^+$ T cells, $CD45RA^+$ and $CD45RO^+$ T cells ($CD4^+$ and $CD8^+$), and $CD5^+$ T and B cells. Analysis of MS patients' peripheral blood revealed essentially normal levels of total T, B, and NK cells. In agreement with results obtained by other investigators, it was found that MS patients had an increased CD4/CD8 ratio, primarily due to a decrease in $CD8^+$ T cells. MS patients were found to have a significantly decreased level of suppressor precursor ($CD28^-$) $CD8^+$ T cells compared with that of controls but to have normal levels of cytotoxic ($CD28^+$) $CD8^+$ T cells. These data indicate that MS patients do not have a general decrease in $CD8^+$ T cells but that they have a specific decrease in the suppressor precursor subset only and normal levels of cytotoxic $CD8^+$ T cells. MS patients also had a significant increase in memory ($CD45RO^+$) $CD4^+$ T cells and displayed a trend towards a decrease in naive ($CD45RA^+$) T cells in the peripheral blood.

Multiple sclerosis (MS) is an autoimmune-based, demyelinating disease affecting the white matter of the central nervous system and brain. MS is common in the United States, with over 300,000 cases annually, primarily affecting young adults. The initial cause of MS is unknown. Clinically MS is characterized by recurrent attacks and exacerbations of neurologic dysfunction leading to progressive physical disability (7, 27). Recent studies have characterized immunoregulatory abnormalities in MS. MS lesions in the brain and central nervous system are characterized by specific immune recognition and activation of T cells, B cells, and macrophages (8). The primary, although not unique, autoantigen appears to be the neural myelin sheath, specifically, the myelin basic protein component (6, 24). Since the disease manifests itself in central nervous system plaques, significant pathology in other organs is limited; however, immune abnormalities are detectable in the peripheral blood. The presence of myelin basic protein-specific T cells has been demonstrated in the peripheral blood (6, 24), and MS patients have been found to have elevated ratios of $CD4^+$ and $CD8^+$ cells (9, 21, 28). Taking into account the well-documented immunoregulatory abnormalities in MS, we have performed a comprehensive peripheral blood immunophenotype survey of MS patients and normal controls. Specifically, the levels of T cells, B cells, monocytes, $CD4^+$ and $CD8^+$ T cells, memory and naive $CD4^+$ and $CD8^+$ T cells, suppressor precursor and cytotoxic $CD8^+$ T-cell subsets based on CD28 expression, and early ($CD69^+$)-activated and late ($HLA\text{-}DR^+$)-activated T cells were determined.

Sixteen patients in various stages of clinically defined MS (25) were selected for this study. Patient information on age, sex, clinical status, and current medication(s) is presented in Table 1. Disease severity was determined according to the expanded disability status scale (12). MS exacerbation was defined as the occurrence of a symptom or symptoms of neurological dysfunction not previously present and lasting for at least 24 h (19). Patient blood specimens were drawn approximately 2 to 4 months after the onset of symptoms present during the current exacerbation. Seventeen healthy Tampa General Hospital employees (nine females and eight males) were surveyed as age-matched controls for the study. There were no differences between males and females in the control group for any of the markers investigated. Venipuncture was performed and blood specimens were collected both from MS patients and from normal controls in an EDTA (purple top) anticoagulant tube.

Individual peripheral blood immunophenotype determinations for MS patients were assessed with a FACScan five-parameter flow cytometer (Becton Dickinson, Mountain View, Calif.). The antibody combination design of the flow cytometry panel is outlined in Table 2. The flow cytometry was set up with appropriate electronic compensation for spectral overlap of the light emitted by the different fluorochromes. The lymphocyte cluster was identified by forward versus side (90°C) light scatter, confirming the accuracy of the lymphocyte gate by using a combination of the CD45 (pan leukocyte) and CD14 (monocyte-specific) antibodies to verify no debris or monocyte contamination of the lymphocyte cluster. The lymphoid cells were then gated for immunofluorescence analysis; only the lymphoid cells were included in the data analysis. Thresholds for positivity were set with isotype-matched control antibodies. For analysis, 50 μl of peripheral whole blood was stained along with the manufacturer's suggested amount of fluorescein isothiocyanate, phycoerythrin, or peridinin chlorophyll protein- or phycoerythrin/Cy5-labeled monoclonal antibodies (Table 2) in 100 μl of phosphate-buffered saline (PBS; pH 7.0). The mixtures were incubated on ice for 15 min, after which 3 ml of

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TABLE 1. Sex, age, clinical status, extended disability status scale score, and current medication(s) for MS patients

Patient	Sex[a]/age	Clinical status[b]	EDSS score[c]	Medication(s)[d]
1	F/35	EX	5.0	PX, Z
2	F/37	EX	5.5	X, SY
3	M/43	EX	6.0	None
4	F/52	EX	6.5	B, P, DT
5	F/44	EX	4.5	Z, DN
6	F/44	EX	7.5	M, B, T, PB
7	F/50	EX	8.0	DT
8	F/54	CP	6.5	BS, Q, DL, DT
9	M/47	CP	8.0	B, EL, SY, TH, DA, HC
10	F/43	EX	6.0	B, SY
11	M/40	CP	8.0	B, VA
12	F/49	EX	6.0	BS
13	F/35	EX	3.0	None
14	M/33	EX	2.0	None
15	F/49	EX	2.5	None
16	F/32	EX	6.0	BS

[a] F, female; M, male.
[b] EX, patient believed to be in current MS exacerbation; CP, patient displaying chronic progressive MS symptoms.
[c] EDSS, expanded disability status scale.
[d] X, Xanax (alprazolam); PX, Paxilon (methazole); Z, Zantac (ranitidine hydrochloride); P, Prozac (fluoxetine); B, baclofen; DT, Ditropan (oxybutynin chloride); DN, Donnatal; PB, phenobarbital; M, methylmednisolone; T, Tenuate (diethylpropion hydrochloride); BS, Betaseron ($beta_{ser}$ interferon); Q, vitamin B_{12}; DL, Dalmane (flurazepam hydrochloride); TH, theophylline; HC, hydrocortisone; EL, Elavil (amitriptyline hydrochloride); DA, Dantrium (dantrolene sodium); SY, Symmetrel (amantadine hydrochloride); VA, Valium (diazepam).

0.85% ammonium chloride was added; the mixtures were then incubated in the dark for 5 min to lyse the erythrocytes, washed three times with 3 ml of PBS, and resuspended in 0.5 ml of PBS with 1% paraformaldehyde. Analysis was performed with Lysys-II software.

Flow cytometry analysis revealed essentially normal percentages of T, B, and NK cells in the peripheral blood of MS patients compared with the percentages in the peripheral blood of controls (Fig. 1). It has been well documented that MS patients have an elevated CD4/CD8 ratio in both their peripheral blood (9, 21, 28) and their cerebrospinal fluid (11, 14). Our analysis of T-cell subsets further confirmed those earlier observations that MS patients indeed have a significant elevation in the peripheral blood $CD4^+/CD8^+$ T-cell ratio. In our study, the elevated ratio was found to be due primarily to a significant decrease in $CD8^+$ cytotoxic and suppressor T cells (mean percentages of $CD8^+$ T cells: for MS patients, 21.8%; for controls, 28.5%; $P < 0.05$) (Fig. 2). Further analysis of percentages of $CD8^+$ T-cell subpopulations based on expression of the CD28 molecule was performed. It has been reported that among $CD8^+$ T cells the cytotoxic subset possesses a $CD3^+$ $CD8^+$ $CD11b^-$ $CD28^+$ phenotype (10), while the suppressor cell precursor subset possesses a $CD3^+$ $CD8^+$ $CD11b^+$ $CD28^-$ phenotype (3, 10, 13, 26). In our study, MS patients had a significant decrease in levels of $CD28^-$ $CD8^+$ suppressor precursor T cells in peripheral blood (mean percentages of CD28 $CD8^+$ cells: for MS patients, 9.9%; for controls, 16.1%; $P < 0.05$), while they maintained normal percentages of $CD28^+$ $CD8^+$ cytotoxic T cells (Fig. 3).

Differences in peripheral blood subsets of memory and naive $CD4^+$ lymphocytes in MS have been determined previously by using the 2H4 (also called Leu18 or CD45RA) surface molecule as a marker for naive $CD4^+$ T cells (1, 16, 17, 29, 30) and the CD29 (also called 4B4) molecule as a marker for memory cells (5, 18, 22, 23). We analyzed percentages of $CD4^+$ memory cells by using the CD45RO molecule as an indicator of memory and the previously described CD45RA molecule as an indicator of naive $CD4^+$ T cells. It was found that MS patients' PB had a significant increase in the percentage of $CD4^+$

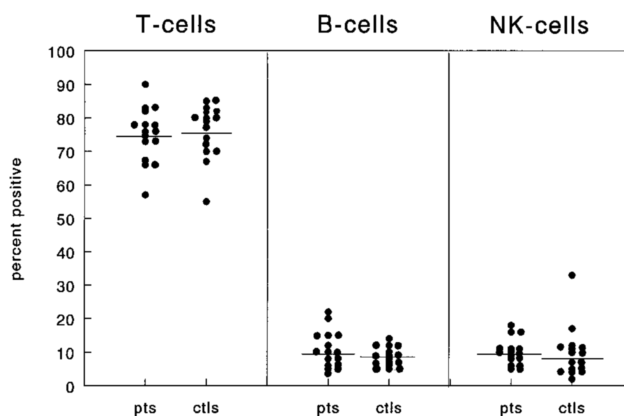

FIG. 1. Scatter plots showing percentages of T, B, and NK cells in the peripheral blood of MS patients (pts) and controls (ctls). Mean percentages are indicated (———).

TABLE 2. Three-color flow cytometry panel for MS patients

Tube	Antibody labeled with[a]:		
	FL1	FL2	FL3
1	CD45[b]	CD14[b]	
2	Control[b]	Control[b]	Control[b]
3	CD3[c]	CD16[c]	CD19[c]
4	CD4[c]	CD8[c]	HLA-DR[b]
5	CD69[b]	CD19[c]	CD5[c]
6	CD45RA[b]	CD45RO[b]	CD4[d]
7	CD45RA[b]	CD45RO[b]	CD8[d]
8	CD28[e]	CD8[c]	

[a] FL1, fluorescence agent 1 (fluorescein isothiocyanate); FL2, fluorescence agent 2 (phycoerythrin); FL3, fluorescence agent 3 (peridinin chlorophyll protein- or phycoerythrin/Cy5-labeled monoclonal antibodies).
[b] Becton Dickinson, Moutain View, Calif.
[c] Caltag, San Francisco, Calif.
[d] Pharmingin, San Diego, Calif.
[e] AMAC, Westbrook, Maine.

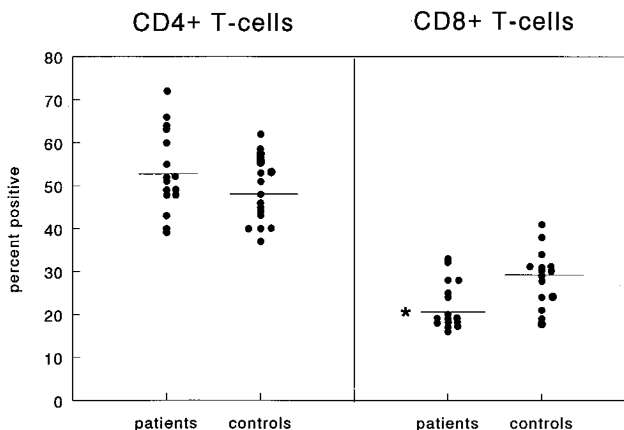

FIG. 2. Scatter plots showing percentages of $CD4^+$ T cells and $CD8^+$ T cells in the peripheral blood of MS patients and controls. Mean percentages (———) and significant differences ($P < 0.05$ by pooled Student's t test) (*) are indicated.

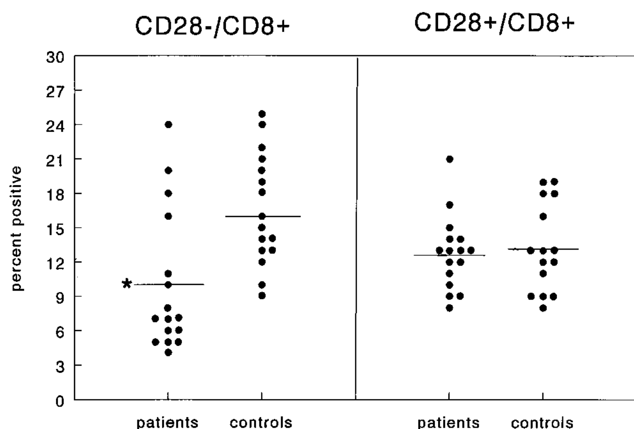

FIG. 3. Scatter plots showing percentages of cytotoxic (CD28$^+$) and suppressor (CD28$^-$) CD8$^+$ T cells in the peripheral blood of MS patients and controls. Mean percentages (———) and significant differences (P < 0.05 by pooled Student's t test) (*) are indicated.

CD45RO$^+$ memory CD4 T cells compared with the percentage in peripheral blood from controls (mean percentages of CD4$^+$/CD45RO$^+$ cells: for MS patients, 36.0%; for controls, 27.2%; $P < 0.05$) (Fig. 4). Patients also had a decreased percentage of CD4$^+$ CD45RA$^+$ naive CD4 T cells, although the difference in our study was not statistically significant. Analysis of CD45RA$^+$ CD8$^+$ and CD45RO$^+$ CD8$^+$ T cells showed no significant differences between MS patients and controls (data not shown).

No significant differences in CD5$^+$ T and B cells were seen between MS patients and controls. Concerning the expression of lymphocyte activation markers CD69 and HLA-DR on lymphocytes, cells bearing these markers never exceeded 3 to 4% in the peripheral blood and no significant differences were seen between MS patients and controls (data not shown).

The peripheral blood immunophenotype changes detected in this study are consistent with those expected in an immune-mediated inflammatory-type disease. The fact that MS pathogenesis is limited to the central nervous system does not preclude detection of changes in the peripheral blood. The increase in the CD4/CD8 ratio is primarily due to a significant

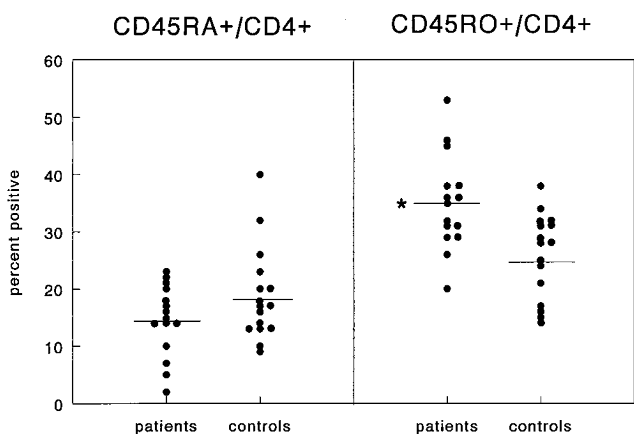

FIG. 4. Scatter plots showing percentages of naive (CD45RA$^+$) and memory (CD45RO$^+$) CD4$^+$ helper T cells in the peripheral blood of MS patients and controls. Mean percentages (———) and significant differences (P < 0.05 by pooled Student's t test) (*) are indicated.

decrease in the CD8$^+$ T-cell population. The decrease in CD8$^+$ T cells in MS has been described previously and was thought to be associated with a decrease in suppressor cell activity (9, 20). This study assessed the presence of the CD28 molecule in peripheral blood lymphocytes to differentiate between suppressor and precursor and cytotoxic CD8$^+$ T cells. The normal function of the CD28 molecule on T cells is to interact with its ligand, the B7 molecule, on antigen-presenting cells. When the T cell recognizes antigen via the T-cell antigen receptor, triggering of the CD28 molecule provides a costimulatory second signal which is required for activation (2, 4). Stimulation of T cells via the antigen receptor in the absence of a secondary costimulatory signal such as CD28 results in T-cell anergy (15). CD28 is one of several T-cell surface molecules capable of delivering the necessary costimulatory signal to produce activation. We found that the CD28$^-$ CD8$^+$ T-cell population was decreased in the blood of MS patients, while the CD28$^+$ CD8$^+$ cytotoxic T-cell population in the peripheral blood was unaffected by disease. In addition, we confirmed other investigators' findings that there is an increase in memory CD4$^+$ T cells in the peripheral blood of MS patients by using the surface expression of the CD45RO molecule. Flow cytometry analysis of blood leukocytes from patients with other inflammatory-type autoimmune diseases will be necessary to determine if the described decrease in peripheral blood CD28$^-$ CD8$^+$ suppressor cells is unique to MS or is found in other such diseases.

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