Analysis of Peripheral Blood Lymphocytes of Relapsing-Remitting Multiple Sclerosis

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ABSTRACT. Peripheral lymphocyte surface antigen subsets were analyzed in 25 patients with multiple sclerosis. These patients were divided into two subgroups of patients in the stable phase and those in relapse. As compared with a normal control group and stable phase group, the relapse group showed decreased $CD4^+CD45RA^+$ cells ($p<0.0001$ and $p<0.05$, respectively) and increased $CD4^+CD45RO^+$ cells ($p<0.005$ and $p<0.01$, respectively). $CD3^+IL\text{-}2R^+$ cells significantly increased during relapse as compared with the stable phase ($p<0.02$). $CD3^+\gamma\,\delta T$ cells decreased in the relapse group as compared with the normal control group ($p<0.05$). No significant changes were observed in the CD4/CD8 ratio, unlike the findings in previous reports. It was confirmed that analysis of peripheral lymphocyte subsets provides sufficient indicators for the determination of the activity of multiple sclerosis. The decreases in $CD4^+CD45RA^+$ cells and $CD3^+\gamma\,\delta T$ cells proved to be a particularly sensitive indicator for the diagnosis of patients who had had only one attack or had not yet been diagnosed, and the increases in $CD4^+CD45RO^+$ cells and $CD3^+IL\text{-}2R^+$ cells were determined to be a sensitive indicator for the prediction of relapse among patients diagnosed as having multiple sclerosis.

Key words: multiple sclerosis — naive cell — CD45 — T cell subset

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system, showing repeated remission and relapse. The etiology of this disease is thought to involve some autoimmune mechanism, but still largely remains obscure. Although symptoms can be improved in many cases by high-dose steroid therapy during an acute relapsing phase,[1,2] its prognosis largely depends on the availability of early detection of relapse based on objective evidence. Relapse is usually predicted by reference to clinical symptoms, magnetic resonance imaging (MRI) findings, and electrophysiological test results. However, areas with abnormal signal intensity may not be demonstrated on MRI if examination is delayed or in repeatedly relapsing cases. Therefore a much simpler routine examination may be required to detect evidence of relapse in clinical cases.

A number of studies have been performed to analyze the peripheral lymphocyte surface antigen subsets in MS. Although an increased CD4/CD8 ratio once attracted keen attention,[3-5] subpopulations of CD4 have been studied extensively in recent years. To date, the CD45 antigen has been determined to have five isoforms. Among these isoforms, $CD4^+CD45RO^+$ T cells, called memory cells, are now thought to have a proliferative response to

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the antigen, by which the cells have been stimulated (helper inducer), whereas $CD4^+CD45RA^+$ T cells, called naive or virgin cells, are thought to be immature cells without such function (suppressor inducer).[6] Since Rose *et al*[7] and Morimoto *et al*[8] reported a decrease in naive cells from peripheral lymphocytes during aggravation of MS, a number of related studies have been published, and such decrease is now thought to be a reliable indicator of the activity of MS. In addition, interleukin-2 receptor (IL-2R) and human leukocyte antigen DR (HLA-DR), expressed on activated lymphocytes, have also been studied with regard to whether they can serve as indicators for an active phase of MS. However, memory cells, another isoform of CD45 antigen, have rarely been studied in MS.[6]

In the present study, peripheral lymphocyte surface antigens were analyzed by two-color flow cytometry using various monoclonal antibodies, to determine whether an active phase of MS occurring in Japan could be identified, and which monoclonal antibodies should be selected and combined from many existing antibodies to obtain the most effective indicator.

SUBJECTS AND METHODS

Subjects

Twenty-five patients with MS of the relapsing remitting type, treated at the Division of Neurology, Kawasaki Medical School from April 1991 to March 1995, were examined. According to Poser's diagnostic criteria,[9] 3, 18, and 4 patients were classified as having laboratory-supported definite MS, clinically definite MS, and clinically probable MS, respectively. Among these patients, 22 relapsed, 28 attacks were observed, and 13 patients were in the stable phase. The patients in relapse, 4 males and 18 females, were aged 41.3 years on average (20-70 years). Those in the stable phase, 1 male and 12 females, were aged 34.4 years on average (19-62 years). Nineteen healthy adults, 10 males and 9 females, aged 33.3 years on average (23-51 years), were used as normal controls. Each relapse of MS was defined as aggravation of MS, categorized as 1 or more on Kurtzke's expanded disability status scale (EDSS).[10] Peripheral blood was sampled during a very early period of relapse.

Methods

Heparinized whole venous blood was reacted with various monoclonal antibodies. The actual procedures used were as follows. After 20 μl each of two kinds of monoclonal antibodies were placed in a Falcon tube, 100 μl of heparinized venous blood was added to the tube, gently stirred, and then incubated at room temperature in the dark for 15 minutes. FACS Lysing Solution® (Becton Dickinson Co., Ltd.), diluted 10 times, was added at 2 ml to the tube, stirred for three seconds in vortex, incubated at room temperature in the dark for 10 minutes, and then centrifuged at 1,300 rpm for five minutes at room temperature. The supernatant was removed, and 3 ml of CELL WASH® (Becton Dickinson Co., Ltd.) was added to the pellet, stirred for three seconds in vortex, and then centrifuged at 1,200 rpm for five minutes at room temperature. After the supernatant was discarded, 1 ml of CELL WASH® was added, gently stirred, and stored in a cool dark place. Measurement was performed within two hours after sample preparation; namely, 10,000

lymphocytes were counted using FACS tar® (Becton Dickinson Co, Ltd). The monoclonal antibodies (moAbs) used, which were all obtained from Becton Dickinson Co, Ltd, were anti-Leu 2a (CD8) moAb, anti-Leu 3a (CD4) moAb, anti-Leu 18 (CD45RA) moAb, anti-Leu 15 (CD11b) moAb, anti-Leu 4 (CD3) moAb, anti-Leu 45RO (CD45RO) moAb, anti-interleukin-2 receptor (IL-2R) (CD25) moAb, anti-HLA-DR moAb, and anti-γST-cell receptor (TCR) moAb. The combinations of the moAbs examined are shown in Table 1. Statistical analysis was performed with Mann-Whitney's U test.

TABLE 1. Combination of monoclonal antibodies

	FITC	PE
1	HLA-DR	Leu 4
2	Leu 2a	Leu 3a
3	Leu 2a	Leu 15
4	Leu 18	Leu 45RO
5	IL-2R	Leu 4
6	Leu 18	Leu 3a
7	Leu 3a	Leu 45RO
8	γ δ-TCR	Leu 4

FITC : fluorescein isothiocyanate
PE : phycoerythrin

RESULTS

The $CD4^+CD45RA^+/CD4^+$ ratios were 0.51 ± 0.10 (mean$\pm$SD) in the normal control (NC) group, 0.33 ± 0.11 in the relapsing MS (Re) group, and 0.45 ± 0.11 in the stable phase (S) group. These results indicate a significant decrease in the Re group as compared with the NC and S groups ($p<0.0001$ and $p<0.05$, respectively) (Fig 1). The $CD4^+CD45RA^-/CD4^+CD45RA^+$ ratios were 1.06 ± 0.43 in the NC group, 2.58 ± 1.90 in the Re group, and 1.63 ± 1.31 in the S group, indicating a significant increase in the Re group as compared with the NC and S groups ($p<0.0001$ and $p<0.05$, respectively). The percent of $CD4^+CD45RA^+$ cells in relation to all lymphocytes was $19.88\pm6.94\%$ in the NC group, $12.07\pm5.54\%$ in the Re group, and $17.54\pm7.95\%$ in the S group. These percentages indicated a significant decrease in the Re group as compared with the NC and S groups ($p<0.001$ and $p<0.05$, respectively).

The $CD4^+CD45RO^+/CD4^+$ ratios were 0.57 ± 0.11 in the NC group, 0.68 ± 0.14 in the Re group, and 0.51 ± 0.16 in the S group. These findings showed a significant increase in the Re group as compared with the NC and S groups ($p<0.01$ and $p<0.01$, respectively). The $CD4^+CD45RO^-/CD4^+CD45RO^+$ ratios were 0.85 ± 0.36 in the NC group, 0.56 ± 0.42 in the Re group, and 1.15 ± 0.69 in the S group, indicating a significant decrease in the Re group as compared with the NC and S groups ($p<0.005$ and $p<0.01$, respectively) (Fig 2). There were no significant differences in the percent of $CD4^+CD45RO^+$ cells in relation to all lymphocytes among the three groups. The $CD45RA^+/CD45RO^+$ ratios were 2.23 ± 0.74 in the NC group and 1.73 ± 1.01 in the Re group. There was a significant decrease in the Re group ($p<0.02$).

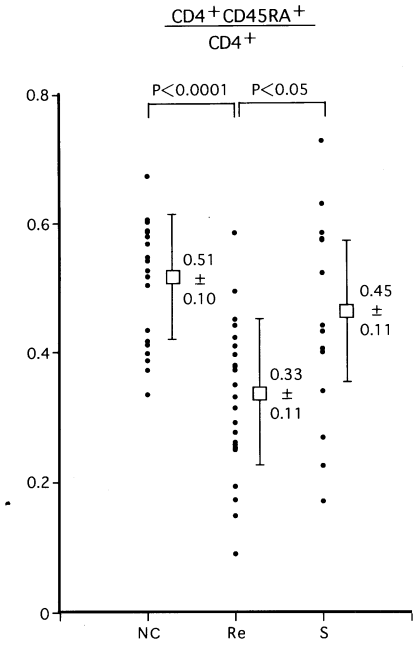

Fig 1. The mean and standard deviation are expressed as open square and vertical bars, respectively.
NC : normal control group
Re : relapsing MS group
S : stable MS group

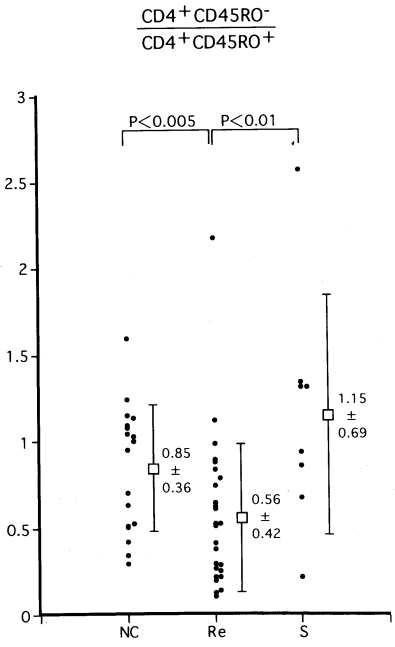

Fig 2. The mean and standard deviation are expressed as open square and vertical bars, respectively.
NC : normal control group
Re : relapsing MS group
S : stable MS group

The $CD3^+CD25^+/CD3^+$ ratios were 0.04 ± 0.01 in the S group and 0.06 ± 0.03 in the Re group, and there was a significant increase in the Re group as compared with the S group ($p<0.02$). The percent of $CD3^+CD25^+$ cells in relation to all lymphocytes was $2.38\pm0.86\%$ in the S group and $4.23\pm2.36\%$ in the Re group, indicating a significant increase in the Re group ($p<0.02$) (Fig 3). The percent of $CD3^+\gamma\ \delta T$ cells in relation to all lymphocytes was $6.07\pm3.48\%$ in the NC group, $4.95\pm6.34\%$ in the Re group and $3.70\pm1.66\%$ in the S group. There was a significant decrease in the Re group as compared with the NC group ($p<0.05$) (Fig 4).

There were no significant differences in the CD4/CD8 ratio, $HLA\text{-}DR^+$ cells, or the CD8/CD11b ratio between the NC, Re, and S groups (Table 2).

TABLE 2. Result

	NC	Re	S	p-value
$CD3^+HLA\text{-}DR^+/CD3^+$	0.14 ± 0.07	0.22 ± 0.15	0.16 ± 0.09	ns
$CD3^+HLA^-DR^+(\%)$	9.39 ± 0.97	14.04 ± 10.77	10.55 ± 1.86	ns
CD4/CD8	1.55 ± 1.00	1.44 ± 0.92	1.33 ± 0.43	ns
$CD8^+CD11b^-/CD8^+CD11b^+$	2.33 ± 0.93	3.44 ± 2.65	3.77 ± 3.41	ns

Data are mean $\pm$S NC: normal control group
Re: relapsing MS group
S: stable MS group
ns: no significance

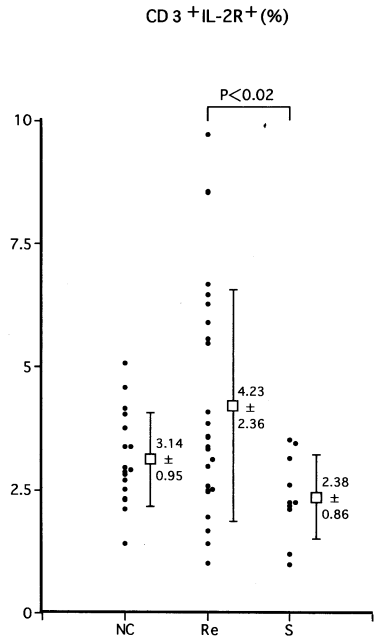

Fig 3. The mean and standard deviation are expressed as open square and vertical bars, respectively.
NC: normal control group
Re: relapsing MS group
S: stable MS group

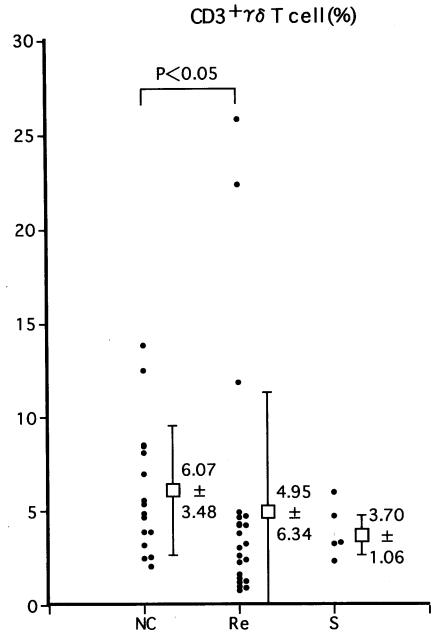

Fig 4. The mean and standard deviation are expressed as open square and vertical bars, respectively.
NC : normal control group
Re : relapsing MS group
S : stable MS group

DISCUSSION

Several reports on peripheral lymphocyte surface antigens in relation to MS have been published. Many investigators have reported a non-specific increase in the CD4/CD8 ratio associated with a decrease in $CD8^+$ cells during active phases,[3-5] but some others have observed no such decrease.[11-13] In recent years, it has been possible to perform more detailed analysis through fluorescent double staining of subpopulations of CD4 cells. Morimoto *et al*[8] reported a significant decrease in peripheral suppressor inducer T cells during aggravation of MS in 1987. Supplementary studies have disclosed comparable results.[7,14-18] From the results of these studies, it has been inferred that the non-specific decrease in CD8 cells reported previously results from a decrease in $CD4^+CD45RA^+$ cells, suppressor inducer T cells.[15] In the present study, I also confirmed that $CD4^+CD45RA^+$ cells decreased and $CD4^+CD45RO^+$ cells increased during a relapse of MS, as reported by Morimoto *et al*, indicating a state of strong sensitization.

In the present study, $IL\text{-}2R^+$ cells, which are expressed on activated lymphocytes, were found to significantly increase in the relapsing MS group, as compared with the stable MS group. Corrigan *et al*[14] reported that $IL\text{-}2R^+$ cells did not reflect the activity of the disease, whereas Selmaj *et al*[19] stated that the increase in $IL\text{-}2R^+$ cells was specific to MS. Ruutiainen *et al*[20] also stated that $IL\text{-}2R^+$ cells increased during aggravation phases, and decreased as the patients began to respond to treatment. Scolzzi *et al*[21] observed increases

in both HLA-DR$^+$ cells and IL-2R$^+$ cells in MS. In addition, it has been reported that serum soluble IL-2R increases during relapsing phases of MS.[22] In the present study, IL-2R$^+$ cells significantly differed in number between relapse and stable MS groups. This seems to indicate that the IL-2R$^+$ cell can be useful for estimating the relapse of MS in patients diagnosed as having MS and, therefore, it can serve as a valuable indicator during follow-up of the course of stable MS.

To date, it has been clinically determined that administration of interferon (IFN)-alpha and -beta improves the symptoms of MS, but IFN-gamma worsens those symptoms.[23,24] It has also been reported that IFN-gamma increases about two weeks before aggravation of MS.[25] CD4$^+$CD45RO$^+$ cells are major IFN-gamma-producing cells, and have been shown to stimulate the production of IL-2.[26,27] From these results, it can be concluded that a decrease in CD4$^+$ CD45RA$^+$ cells, an increase in CD4$^+$CD45RO$^+$ cells, and an increase in IL-2R$^+$ cells represent a series of consistent immune responses.

There have been very few studies of $\gamma\ \delta$T cells in MS. Droogan *et al*[28] showed that, in cerebrospinal fluid (CSF), $\gamma\ \delta$T cells were significantly lower than in a non-inflammatory control, but, in peripheral blood, there were no significant differences. In the present study, the number of $\gamma\ \delta$T cells was significantly lower in the Re group. Apoptosis of $\gamma\ \delta$T cells is induced in the presence of recombinant IL-2.[28] IL-2 and soluble IL-2R increase in MS sera.[29] The result in the present study is compatible with these findings.

As previously noted, many investigators have reported a non-specific increase in the CD4/CD8 ratio in MS, but others have reported no such significant increase, as was the case in the present study. This may indicate that a decrease in suppressor inducer cells does not always result in a quantitative decrease in CD8 cells, but does result in a qualitative change.[16] In addition, it has been pointed out that suppressor/cytotoxic T cells may show different CD4/CD8 ratios according to the monoclonal antibodies used.[30,31] These may be the reasons for no decrease in the CD4/CD8 ratio in the present study.

HLA-DR$^+$ cells did not differ among the three groups examined in the present study. This does not agree with the ability of IFN-gamma to express HLA-DR antigen on the peripheral lymphocyte surface,[32] if administered, or with an increase in HLA-DR$^+$ cells during relapse of MS, as reported by Scolzzi *et al*,[21] but it does agree with the results obtained by Corrigan *et al*.[14] Salmaggi *et al*[33] reported a decrease in HLA-DR$^+$ cells in MS. Therefore, changes in the peripheral HLA-DR$^+$ cells in MS are still the subject of much controversy.

The effect of steroid therapy was not considered in the present study. Reports of the effects of steroid therapy on lymphocyte surface antigens have not been consistent; namely, a number of reports disclosed some changes in peripheral lymphocyte surface antigens,[34–36] but some others detected no such changes.[20] This issue should be further studied.

Since an increase in cells in the CSF is small in MS, only a few reports on CSF lymphocytes have been published. Some authors compared CSF lymphocytes with peripheral lymphocytes, and found a more remarkable decrease in naive cells in CSF.[37–39] Scolozzi *et al*[21] found a higher expression rate of IL-2R$^+$ cells in CSF as compared with peripheral blood. Further

studies on CSF with the accumulation of more data on lymphocyte surface antigens should provide useful clinical information.

Although MRI and electrophysiological findings are useful for the prediction of relapses of MS, it is not always possible to do these examinations for various reasons. Although high-does steroid therapy is often initiated based on aggravation of EDSS in routine physical examinations before various tests provide results, it is desirable to have various parameters that can be used as indicators for relapse, even for the purpose of preventing excessive steroid administration. Although a decrease in $CD4^+CD45RA^+$ cells is not specific to MS, being also observed in active systemic erythematodes and human T lymphotrophic virus-I-associated myelopathy,[40,41] this change in peripheral lymphocyte surface antigens was found to be useful in the present study to ensure there was a relapse, and then to initiate treatment. Since there are numerous human lymphocyte surface marker antibodies, it is difficult to select a suitable combination of antibodies. From the results of the present study, it was determined that the decrease in $CD4^+CD45RA^+$ and $CD3^+\gamma\ \delta^+$ peripheral lymphocytes and the increases in $CD4^+CD45RO^+$ cells and $CD3^+$IL-$2R^+$ cells can serve as useful indicators for relapse, and an examination limited to these antibodies also has an economic merit. It was particularly noteworthy that the decrease in $CD4^+CD45RA^+$ cells, which most significantly differed between the normal control and MS groups, is a useful indicator before definite diagnosis of MS is made. Increases in $CD4^+CD45RO^+$ cells and $CD3^+$IL-$2R^+$ cells should be carefully observed in patients diagnosed as having MS to detect a relapse during their clinical courses. It was concluded that for a combination of CD4 and CD45RA, the $CD4^+CD45RA^+/CD4^+$ ratio or $CD4^+CD45RA^+/CD4^+CD45RA^+$ ratio, and a combination of CD4 and CD45RO, the $CD4^+CD45RO^-/CD4^+CD45RO^+$ ratio are more sensitive, because a significant difference may not be detected by comparing the frequencies of positive cells alone, but can be demonstrated by comparing the ratios of positive cells to negative cells.

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