Peripheral blood T lymphocyte changes in multiple sclerosis: a marker of disease progression rather than of relapse?

AJ THOMPSON, J BRAZIL, CA WHELAN, EA MARTIN, M HUTCHINSON, C FEIGHERY

From the Department of Neurology, Adelaide and St Vincent's Hospital and Department of Immunology, St James's Hospital, Dublin, Ireland

SUMMARY A serial study of peripheral blood T lymphocytes in 27 patients with clinically definite multiple sclerosis and 11 healthy controls was carried out over a 12 month period. This showed that contrary to many previous reports, relapses were not consistently associated with reduced numbers of peripheral blood suppressor T lymphocytes or any other T cells. Persistently low T cells numbers, including both the helper and suppressor T cell subsets, were, however, associated with disease activity as measured by the development of increased disability during the course of the study. This was true both for the patients with relapsing/remitting disease and those with progressive disease. The importance of carrying out a serial study was emphasised by the consistent and significant differences that were detected between individuals in both the control and the patient groups. A serial study is the most reliable means by which clinical events can clearly be correlated with laboratory estimations. The association in this study between the development of increased disability and persistently low levels of peripheral blood T lymphocytes suggests that both may be related to the underlying disease process in multiple sclerosis.

Quantitative abnormalities of peripheral blood lymphocytes in patients with multiple sclerosis were first described in 1975.^{1,2} With the introduction of monoclonal antibodies these changes were confirmed and a close association between reduced suppressor T cells and relapses was demonstrated.^{3,4} This finding was supported by a number of later studies,^{5–7} some of which found an even more marked reduction in patients with chronic progressive multiple sclerosis.^{8,9} Recent reports have, however, failed to confirm the relationship between these changes and disease activity in multiple sclerosis.^{10,11} While these differences in results may be explained in part by varied methodology,^{12–15} it is clear that studies involving single determinations in individual patients should be interpreted with caution and there is an obvious need for serial investigation of peripheral blood lymphocytes in multiple sclerosis. There have been a limited number of such studies^{16–18} and these have been

unable to demonstrate a straightforward relationship between changes in T cells and relapse in multiple sclerosis.

The implications of such a relationship for the pathogenesis and potential treatment of multiple sclerosis provided the stimulus for a serial study involving 27 patients with clinically definite multiple sclerosis and 11 healthy controls over a 12 month period.

Patients, materials and methods

The study groups were composed of 11 healthy laboratory personnel and 27 patients with clinically definite multiple sclerosis,^{19,22} with relapsing/remitting disease and five who had entered the progressive phase. Baseline data on these two groups are shown in table 1. Patients were selected from those who presented in relapse and who agreed to participate in the study. Patients and controls were seen every six weeks over a 12 month period and blood samples were taken between 8.30 and 11.00 am. At each session a history was taken from each patient and the relevant examination carried out. If a patient developed new symptoms or deteriorated in any way between sessions, an immediate appointment was made and where possible the patient was seen every 2 weeks for six weeks. No patient was on steroids

Address for reprint requests: Dr Alan Thompson, Department of Neurology, St Vincent's Hospital, Dublin 4.

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Table 1 Baseline data on study groups

	No of individuals	No of samples	M/F	Mean age in years (range)
Controls	11	92	4/7	28.3 (24–35)
Relapsing/remitting multiple sclerosis	22	206	4/18	38.4 (23–65)
Progressive multiple sclerosis	5	41	1/4	44.4 (27–56)

or any other immunosuppressive therapy for at least six months prior to sampling.

Relapse was defined as a period of neurological dysfunction of at least 24 hours duration with objective evidence on examination, and acute relapse as being within two weeks of the onset of such symptoms. Remission was defined as a definite improvement in symptoms and/or signs lasting a minimum of one month²⁰ while progressive disease was defined as a steady deterioration in neurological status without remission for a period of six months. Disability was measured using the Kurtzke Disability Status Scale.²¹

Venous blood (10 ml) was collected into heparinised evacuated tubes. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation, washed twice in phosphate-buffered saline (PBS) and adjusted to a concentration of $5 \times 10^6 \text{ ml}^{-1}$. 100 μl aliquots were reacted with

the appropriate monoclonal antibody; Leu 4 to measure the total T cells, Leu 3a the helper subset, and Leu 2a, OKT 8 and OKT 5 to measure the suppressor subset. (The Leu reagents were obtained commercially from Becton-Dickinson, OKT 8 was purchased from Ortho Diagnostic Systems and OKT 5 was obtained as a gift from Dr Gideon Goldstein of Ortho). The cells were incubated for 30 min at 4°C, washed twice in PBS and then reacted with fluoresceinated goat anti-mouse IgG (Tago) for an additional 30 min. Two hundred cells were counted within 24 hours of sampling using a Leitz Dialux UV microscope. All slides were examined without knowledge of the monoclonal antibody or the patient under scrutiny, to avoid bias. Differential white cell counts were performed on all samples and the mononuclear cell count determined. This allowed the results to be converted from percentages to absolute

Fig 1 Comparison of results obtained from healthy control individuals representing the extremes of normal variation in total mononuclear cell count and in the proportions identified at T cells, helper T cells, or suppressor T cells. Stable differences in the helper/suppressor T cell ratio are also included.

numbers.

A reduction in T cells or T cell subsets of greater than two standard deviations from the mean of the controls values were regarded as significant. Statistical analysis was carried out using the non-parametric Wilcoxon Rank Sum Test and Wilcoxon Test for Pair Differences.

Results

Controls

Ninety-two blood samples were obtained from 11 controls at six week intervals over a 12 month period (table 1). Consistent differences were found amongst controls in the absolute numbers of cells identified as T lymphocytes or as helper or suppressor T cells. These differences resulted from variations both in the total monoclonal cell count and in the proportions of T cells or their subsets. This is illustrated by the comparison of results obtained from control individuals representing the extremes of normal variation (fig 1). Significant consistent differences were also found in the ratio of helper T cells to suppressor T cells. None of these differences was attributable to either the age or the sex of the control. Two of the controls had consistently low numbers of T cells throughout the study for no apparent reason. Of the 92 samples obtained from the controls, only three samples yielded suppressor T cells values (measured by Leu 2a) less than two standard deviations below the mean

of all controls and these were obtained from one of the two individuals with consistently low levels. There was no evidence of any seasonal variation in these results.

Multiple sclerosis patients

Details of the multiple sclerosis patients studied are outlined in table 2. Eighteen of the 22 patients with relapsing/remitting disease suffered a total of 38 relapses while four patients suffered no relapses. Twenty two of these episodes were studied in the acute phase (within 2 weeks of the onset of symptoms). Five patients in this group were more disabled by the end of the study period as a result of residual neurological deficits following relapse.

In the relapsing/remitting patients, the reduction in the suppressor cells was greatest in cells identified by the monoclonal antibody Leu 2a (table 3). Measurement of suppressor cells with a second antibody, OKT 8, also revealed significantly fewer cells in the multiple sclerosis patients but the difference was less marked. The difference between these two markers was statistically significant (Leu 2a- $0.29 \times 10^9/l$ versus OKT 8- $0.39 \times 10^9/l$, $p < 0.01$). Surprisingly, the subset of suppressor T cells recognised by a third antibody, OKT 5, showed no difference between the patients and controls although it did recognise fewer cells in the controls than either of the other two anti-

Table 2 Patient data

Patient	Sex	Age (years)	Duration of multiple sclerosis (years)	Relapse in study	Kurtzke score on	
					Entry	Exit
Relapsing/remitting multiple sclerosis						
A	F	54	1	3	0	6
B	F	65	21	2	0	3
C	M	36	6	2	3	4
D	F	40	7	3	2	3
E	F	34	12	2	2	3
F	F	31	9	5	4	4
G	F	58	26	1	2	2
H	M	28	4	1	2	2
I	M	27	1	4	1	1
J	F	27	1	2	1	1
K	F	32	1	2	1	1
L	F	29	8	2	1	1
M	F	23	4	2	1	1
N	F	41	9	2	1	1
O	F	40	1	1	1	1
P	F	44	6	2	1	1
Q	F	57	4	0	1	1
R	F	30	1	1	0	0
S	F	41	2	1	0	0
T	M	29	7	0	0	0
U	F	39	6	0	0	0
V	F	25	8	0	0	0
Progressive multiple sclerosis						
W	F	27	8	—	6	8
X	F	52	22	—	4	6
Y	F	56	16	—	3	4
Z	M	44	13	—	3	4
AA	F	43	6	—	3	3

Table 3 Cumulative mononuclear cell counts and lymphocyte subpopulations in controls and multiple sclerosis patients ($\times 10^9/l$)

	MNC	Leu 4	Leu 3a	Leu 2a	OKT 8	OKT 5
Controls (11)	$2.19 \pm 0.39^*$	1.29 ± 0.33	0.88 ± 0.27	0.48 ± 0.16	0.52 ± 0.17	0.32 ± 0.11
Relapsing/remitting multiple sclerosis (22)	2.16 ± 0.49 NS	1.02 ± 0.24 $p < 0.02^\dagger$	0.73 ± 0.18 NS	0.29 ± 0.12 $p < 0.01$	0.39 ± 0.15 $p < 0.05$	0.31 ± 0.13 NS
Progressive multiple sclerosis (5)	1.92 ± 0.49 NS	0.83 ± 0.21 $p < 0.01$	0.70 ± 0.26 NS	0.19 ± 0.06 $p < 0.01$	0.26 ± 0.08 $p = 0.02$	0.18 ± 0.06 $p < 0.02$

*Mean $\pm$ standard deviation.

† Significance of difference from controls.

NS = not significant.

Table 4 Comparison of lymphocyte subsets in acute relapse with the results of the same twelve patients in remission ($\times 10^9/l$)

	Leu 4	Leu 3a	Leu 2a	OKT 8
Data obtained in acute relapse (28 samples)	$0.90 \pm 0.49^*$	0.72 ± 0.37	0.25 ± 0.21	0.32 ± 0.19
Data obtained in remission (51 samples)	0.98 ± 0.27	0.73 ± 0.25	0.28 ± 0.13	0.31 ± 0.14

*Mean $\pm$ SD.

bodies. There were also reductions in both the total and helper T cell populations although the change in the latter subset was not statistically significant. All of these changes occurred in the absence of any detectable change in the total mononuclear cell count and were also apparent when the results were expressed as percentages.

Relationship between changes in lymphocyte subpopulations and relapse

The association between changes in total T cells and particularly the suppressor T cell subset and relapses was examined in those patients seen in acute relapse. Twelve patients had 28 blood samples taken within 2 weeks following the onset of symptoms during a total

Fig 2 Suppressor T cells measured by Leu 2a in patients with relapsing/remitting and progressive multiple sclerosis. ● = samples not associated with relapse, ▼ = samples taken within two weeks prior to the onset of relapse, and ▲ = samples taken within two weeks following onset. The mean for each individual is indicated by a bar.

of 22 relapses. The data obtained from these patients were compared with data obtained from the same patients when they were clearly in remission (table 4). The patients were used as their own controls to allow for any consistent differences between individuals similar to those seen in the controls. This comparison showed no differences in any of the parameters examined, including suppressor T cells. Thus, although T cells and particularly suppressor T cells are reduced in relapsing and remitting disease, they are not associated with relapses *per se*. The lack of association between reductions in suppressor T cells and individual relapses was confirmed by examining the results from each patient (fig 2). This showed that relapses were associated with both high and low levels of suppressor T cells and furthermore that low levels occurred in the absence of relapse. This was true whether the lymphocyte numbers were expressed as absolute numbers or percentages.

The patterns of variation over 12 months in individual patients in the number of suppressor T cells (identified using Leu 2a) in relation to disease activity were examined. These are illustrated by a number of examples in fig 3. Eight patients had suppressor T cells values within the normal range throughout the study (normal = control mean $\pm$ 2 SD) and three of these had no clinically apparent disease activity dur-

ing this period. The results from one of these patients are illustrated in fig 3A. The remaining five patients whose suppressor T cells remained within the normal range throughout the study suffered at least one relapse and six of these relapses were studied within two weeks of onset. The results from one of these patients are illustrated in fig 3B. Complete recovery was seen after each relapse and none of these patients became more disabled.

Episodically reduced suppressor T cells were observed in eleven patients. In two of these each relapse was associated with reduced levels of suppressor T cells. In a further two patients, reduced suppressor cells were only found in a proportion of relapses (illustrated by the results of one patient in fig 3C). Reduced suppressor T cells were found in both relapse and remission in four patients (illustrated in fig 3D) while in the remaining three patients, these reductions occurred in remission but not in relapse.

Three patients had persistently low levels of suppressor T cells throughout the 12 months of the study (the results of one patient are illustrated in fig 3E). No further reduction in suppressor T cells occurred in these patients during their relapses. All three of these patients made incomplete recoveries from these relapses and developed increased disability during the course of the study.

Development of disability in relapsing/remitting multiple sclerosis

Five patients with relapsing/remitting multiple sclerosis developed increased disability during the course of the study as a result of residual deficits from their relapses. The T cell subsets in these five patients were compared with the remaining 17 patients in the relapsing/remitting group (table 5). There was a highly significant reduction in the total T lymphocyte population in patients who developed increased disability evident in both the helper and suppressor subsets. There was also a reduction in the total mononuclear cell count in the group with increased disability. The reductions in the numbers of T cells were not solely attributable to the reduction in mononuclear cells because there were also significant reductions in the proportions of these cells identified as T cells. Although the patients who suffered increased disability were generally older (45.8 ± 13.3 versus 37.7 ± 11.7 years), had disease of longer duration (9.4 ± 7.6 versus 5.8 ± 6.0 years) and had slightly more relapses in the study (2.4 ± 0.5 versus 1.5 ± 1.5), these differences were not statistically significant.

Lymphocyte subpopulations in progressive multiple sclerosis

The cumulative data from patients with progressive

Fig 3 Patterns of changes over 12 months in suppressor T cells measured by Leu 2a in patients with clinically definite multiple sclerosis with and without disease activity. The broken line indicates the control mean less two standard deviations.

Table 5 Cumulative mononuclear cell counts and T cell subsets in relapsing/remitting multiple sclerosis patients suffering increased disability ($\times 10^9/l$)

	MNC	Leu 4	Leu 3a	Leu 2a	OKT 8	OKT 5
Controls (11)	2.19 $\pm$ 0.39*	1.29 $\pm$ 0.33	0.88 $\pm$ 0.27	0.48 $\pm$ 0.16	0.52 $\pm$ 0.17	0.32 $\pm$ 0.11
No change in disability (17)	2.29 $\pm$ 0.49 NS	1.09 $\pm$ 0.24 p < 0.05†	0.80 $\pm$ 0.14 NS	0.34 $\pm$ 0.09 p < 0.02	0.43 $\pm$ 0.12 NS	0.35 $\pm$ 0.12 NS
Increased disability (5)	1.74 $\pm$ 0.11 NS	0.62 $\pm$ 0.16‡ p < 0.05	0.47 $\pm$ 0.14 p < 0.01	0.15 $\pm$ 0.07 p < 0.01	0.22 $\pm$ 0.09 p < 0.01	0.17 $\pm$ 0.04 p < 0.01

*Mean $\pm$ standard deviation.

†Compared to controls (NS = not significant).

‡Compared to no increase in disability.

disease are presented in table 3. Reductions in total T cells and suppressor T cells were observed similar to those seen in patients with relapsing/remitting disease. Four of these five patients became more disabled through the course of the study sufficient to increase their score on the Kurtzke Disability Scale. All five patients in this group had suppressor T cell estimations that were persistently low (illustrated by patients Y and X in fig 2). Figure 3F illustrates the results over 12 months of one patient from this group. The mean lymphocyte levels in patients with progressive disease were similar to those of the relapsing/remitting patients who developed increased disability.

Discussion

This serial study showed no clear association between relapse and reduced peripheral blood T lymphocytes, including suppressor T cells, despite a reduction in T cells indicated by the cumulative data. Reduced T cells, including both helper and suppressor subsets, are however associated with disease activity measured by the development of increased disability in both patients with relapsing/remitting and progressive multiple sclerosis.

The lack of a direct association between reduced T cells and relapse was shown by the similarity between results obtained from the same patients in acute relapse and in remission. This was supported by the multiplicity of patterns detected when serial results from individual patients were examined. Several of these patterns have been described previously.^{17 18 22} Although sub-clinical demyelination²³⁻²⁵ may explain reduced T cells in the absence of clinical symptoms,^{18 22} the occurrence of relapse in the absence of T cell changes is more difficult to explain. It has been suggested that this may occur because these symptoms are not the result of an acute relapse.¹⁸ This was not the case in the present study where demyelination was confirmed by the detection of myelin basic protein in the cerebrospinal fluid.²⁶

The only consistent finding in this serial study was that the development of increased disability in multiple sclerosis patients (whether as a result of incomplete recovery from relapse or of progressive disease) was associated with persistently low levels of total T cells, including both the helper and suppressor subsets. Although reduced numbers of total T cells and suppressor T cells have been convincingly shown in progressive disease in many studies,^{8 9 27} the association between similar changes in relapsing/remitting multiple sclerosis and the development of increased disability has not previously been demonstrated.

The importance of carrying out a serial study (rather than single determinations) to investigate possible changes in T lymphocytes is emphasised by the significant and consistent differences in lymphocyte numbers in different members of the control group. Two of the 11 controls in our study had repeatedly low T cells and suppressor T cells throughout the study for no obvious reason. One of the three controls in Kastrukoff and Paty's serial study¹⁸ similarly exhibited persistently low figures and Compston suggested in a paper subsequent to his serial study¹² that "it may be inappropriate to cite a normal range for the peripheral blood T lymphocytes subpopulations". Any single result from a patient should therefore be compared with that patient's previous figures. This is the only means by which clinical events can be clearly correlated with laboratory estimations.

There have been only a limited number of serial studies in the literature¹⁶⁻¹⁸ and in each case, the authors accept that the relationship between reduced suppressor T cells and relapse is inconsistent and requires further examination. In the largest of these studies, Compston¹⁷ detected reduced suppressor T cells in 25% of patients in the absence of clinical evidence of relapse. In contrast, clinical evidence of relapse in the absence of altered T cells were documented in the other two studies.^{16 18} Both of these studies involved small numbers of patients and controls examined for only six months. Consequently the number of relapses which occurred was significantly

less than in the present study.

The cumulative data in this study demonstrated significant reductions in both the total T cell and suppressor T cell subset in patients with relapsing/remitting multiple sclerosis and to a greater extent in patients with progressive disease. These findings are not attributable to the differences in age between the control population and the patients with multiple sclerosis²⁸ and are in agreement with the majority of single measurement studies.³⁻⁹ In their serial study, Kastrukoff and Paty¹⁸ found no significant difference in cumulative data between patients with relapsing/remitting multiple sclerosis and controls, possibly because of the limited number of controls.

The difficulties in the clinical assessment of disease activity in multiple sclerosis remain unresolved. The development of increased disability possibly provides a more accurate indication of disease activity in multiple sclerosis than the occurrence of relapses because it results from demyelination which is clinically apparent as a relapse or which may remain sub-clinical. Increased disability might also result from an impaired capacity to recover from the demyelination. The association in this study between the development of increased disability and reduced peripheral blood T cells suggests that both may be associated with the same pathological process. The reduction in the number of peripheral blood mononuclear cells, and especially T cells, may in part reflect sequestration of these cells within the central nervous system where they may precipitate demyelination or possibly impede recovery from demyelination. In the early stages of plaque formation in multiple sclerosis, most of the infiltrating cells are T cells of haematogenous origin,²⁹ and a similar situation occurs in experimental allergic encephalomyelitis.³⁰ Although the comparison with multiple sclerosis is uncertain, T lymphocytes have been shown to influence the recovery of mice suffering from virus-induced demyelination.³¹ On the other hand, some of the reduction in peripheral blood T cells recognised by monoclonal antibodies may be more apparent than real because of the failure of these cells to express the cell surface proteins normally used to identify the different T cell subsets.^{32,33}

Irrespective of the role that T cells play in the underlying disease process in multiple sclerosis, there is an obvious advantage in the identification of those patients who are likely to become more disabled in the short term. This may be of relevance for the selection of patients for inclusion in therapeutic trials.

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