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Identification of three FcR-positive T cell subsets (Tγ, Tμ and T$\gamma\mu$) in the cerebrospinal fluid of multiple sclerosis patients

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SUMMARY

Proportions of T cells and T cell subsets, as identified by their Fc receptors (FcR) for IgM and IgG (Tμ and Tγ), were determined in the peripheral blood lymphocyte (PBL) and cerebrospinal fluid (CSF) lymphocyte populations in patients with multiple sclerosis (MS). On average, MS patients had 79% total T cells (62% of which were Tγ, 66% Tμ) in CSF lymphocytes compared to 66% total T cells (30% Tγ, 63% Tμ) in PBL. Normal age- and sex-matched controls PBL had 74% total T cells (20% Tγ, 54% Tμ). By direct observation using an indirect immunofluorescence assay, 41% of the CSF Tγ cells in MS patients bore receptors for IgM; these cells were designated T$\gamma\mu$ and, according to the double-marker analysis, did not seem to correlate with disease stage. In MS PBL, 20% of Tγ cells were T$\gamma\mu$ compared to 9% in the control PBL Tγ population. Thus, MS patients had a higher proportion of total T cells, Tγ cells and T$\gamma\mu$ cells in their CSF than in their peripheral blood and than those populations found in normal control blood. The significance of this T$\gamma\mu$ population for the continuing disease state in MS is discussed.

INTRODUCTION

Among several immunological disorders that occur in patients with multiple sclerosis (MS) are aberrations in the proportions of peripheral blood lymphocyte (PBL) total T cells and the T cell subset defined by its Fc receptor (FcR) binding of IgG (Tγ cells; Ferrarini *et al.*, 1975) as has recently been reported by this laboratory (Merrill *et al.*, 1980) and previously by several others (Sandberg-Wollheim & Turesson, 1975; Traugott, 1978; Naess & Nyland, 1978; Santoli *et al.*, 1978; Huddlestone & Oldstone, 1979). In addition, we have further shown an increased proportion of a third T cell subset in MS PBL compared to normal PBL which bears FcR for both IgG and IgM (T$\gamma\mu$) (Merrill *et al.*, 1980).

Since there is an intact blood-brain barrier in 75% of all MS patients (Schliep & Felgenhauer, 1978) and the resulting pathological condition of demyelination within the white matter of the brain seems to be immunologically mediated (Davison, 1978), the central nervous system (CNS) should be considered a separate and more relevant immunological compartment in which to assess lymphocyte subsets and their functions. Many reports in fact have shown higher proportions of T cells in the CSF of patients than in their peripheral blood (Sandberg-Wollheim &

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Turesson, 1975; Kam-Hansen, Frydén & Link, 1978) with further increase of T cells in CSF during exacerbation (Levinson, Lisak & Zweiman, 1976; Traugott, 1978; Naess & Nyland, 1978).

We report here, as have others, that T cells of MS patients represent a greater proportion of total lymphocytes in their CSF than in their peripheral blood. Compared to normal control blood, the percentages of $T\gamma$ and $T\mu$ cells are elevated in MS blood, without correlation with disease stage. An increased proportion of $T\gamma\mu$ cells in the peripheral blood of MS patients may explain, in part, the elevation of $T\gamma$ and $T\mu$ cells; this is described here as previously (Merrill *et al.*, 1980). We now report further on the three T cell subsets, defined by their FcR, in the CSF lymphocyte population in MS patients.

MATERIALS AND METHODS

Subjects. Twenty-four MS patients seen at the Department of Neurology at either Danderyd Hospital or Karolinska Hospital, Stockholm, were studied. Eleven patients were classified as acute cases of MS undergoing relapses at the time of this study, seven patients as chronic cases having had relapses within the previous 12 months but not at the time of study, and six patients as stable cases having had no relapses for 12 months or more according to the criteria of Schumacher *et al.* (1965). No patients were being treated with steroids at the time of this study, nor had there been such treatment for at least 2 months previously. Age- and sex-matched controls were used in each case except acute patient 4. Peripheral blood samples were drawn in the morning at the same time as the cerebrospinal fluid was taken and all cells were handled throughout the experimentation at room temperature unless otherwise noted.

Cell preparation. Peripheral blood was collected in heparinized tubes and after dilution 1:1 in phosphate-buffered saline (PBS), lymphocytes were separated from red blood cells on Ficoll–Hypaque (FH; Pharmacia, Uppsala, Sweden) (Böyum, 1976). After centrifugation on FH for 30 min at 400 *g*, lymphocytes were washed three times in RPMI 1640 medium. CSF was collected into pre-siliconized glass, conical-bottomed tubes. Immediately after collection, 20% FCS was added to the CSF and the cells gently pelleted at 300 *g* for 10 min and resuspended in RPMI 1640. For removal of macrophages, cells were incubated in the same medium supplemented with 20% foetal calf serum (FCS) in plastic tissue culture dishes (NUNC, Roskilde, Denmark) for 1 hr at 37°C. Removal of monocytes/macrophages was usually 80–90% complete as assessed by non-specific esterase staining (Tucker, Pierre & Jordan, 1977).

E rosettes. T cells were identified by rosetting with sheep red blood cells (SRBC) which had previously been treated with 0·14 M 2-aminoethylisothiouronium bromide hydrobromide (AET; Sigma, St Louis, Missouri) (Saxon, Feldhaus & Robbins, 1976). Briefly, lymphocytes, in RPMI 1640 medium, were mixed with AET-treated SRBC, which had been diluted in SRBC-absorbed, heat-inactivated FCS, to a final ratio of 100:1 SRBC: lymphocyte. The mixture was centrifuged into a pellet at room temperature and incubated for 1 hr at 4°C. After gentle resuspension, the E rosettes formed were counted and the percentage of the total T cells was calculated. As has been reported by others (Levinson *et al.*, 1976), we found it advantageous to aspirate 2/3 of the supernatant off of the CSF cell–SRBC pellet prior to resuspending in order to increase the concentration of cells prior to scoring. The mixture of E rosettes and non-T cells was gently layered onto a FH gradient and the T cells sedimented from the non-T cells (Moretta *et al.*, 1975). A single FH gradient was sufficient to produce a greater than 95% E rosette-positive pellet from the CSF cells. However, for the PBL, the FH gradient was repeated a second time and the second pellet contained usually 95–100% E rosettes. By non-specific esterase staining (Tucker *et al.*, 1977), there were fewer than 1% macrophages in this population. The presence of surface immunoglobulin-positive (SIg) cells was assessed by incubating these cells with a 1:6 dilution of FITC-conjugated $F(ab')_2$ sheep anti-human Ig (National Bacteriology Laboratory, Stockholm, Sweden) for 30 min at 4°C. After washing twice, the cells were viewed under epi-illumination on a Leitz Orthoplan fluorescence microscope. The E rosette-enriched fraction contained less than 3% contamination by SIg-positive cells for PBL T cells and 0% SIg-positive cells in CSF T cells.

The SRBC attached to T cells were lysed by hypotonic shock: a 20 sec incubation with 9 ml of double-distilled water followed by 1 ml of 10× PBS.

Tγ and Tμ rosettes. Antibodies to ox RBC (ORBC) of the IgM and IgG classes were raised in rabbits and the purity of these reagents shown according to previously described techniques (Merrill *et al.*, 1980). The ox RBC–antibody complexes used for identifying T cell subsets according to their FcR for IgM or IgG were prepared according to Biberfeld, Nilsson & Biberfeld (1979).

Tγ and Tμ cells were identified by rosette formation (Moretta *et al.*, 1975). One hundred microlitres of cells (either PBL T cells or CSF T cells) were mixed with 100 μl of ox RBC coated with IgG (ORBCγ) or IgM (ORBCμ) for an ORBC: lymphocyte ratio of 100:1, centrifuged into a pellet at 200 *g* and incubated for 1 hr at 4°C. The pellet was gently resuspended and 200–300 T cells from each tube were counted for rosette formation. A rosette was defined as a lymphocyte with four or more red cells attached. All tests were performed in duplicate. T cells were examined for binding ORBCγ or ORBCμ both on the day the blood or CSF was drawn (designated day 0) and, after incubation overnight at 37°C in RPMI 1640 plus 20% FCS, on the day designated day 1. T cells binding IgM by FcRμ are Tμ, while T cells binding IgG are Tγ. Day-1 T cells were also re-rosetted to ascertain the E rosette purity which was 98±2% for both PBL and CSF T cells. As for E rosetting of CSF cells, aspiration of the supernatant was performed prior to resuspending Tγ or Tμ rosettes in CSF to increase the cell concentration prior to scoring.

Tγμ rosettes. Direct demonstration that T cells bearing FcR for IgG also bear FcR for IgM was accomplished using a double-marker system. T cells from the E rosette-enriched population were first incubated with ORBCγ as above. The resulting Tγ rosettes were then incubated first with 10 mg/ml of aggregated IgM (IgM_{agg}) from a Waldenström's macroglobulinaemia patient (a generous gift from Dr Birger Andersson). The purification of this reagent has been described before (Andersson, Skoglund & Rosén, 1979). Following incubation with IgM_{agg} for 30 min at 4°C, the cells were diluted gently in RPMI 1640 and pelleted at 4°C at 400 *g*. The supernatant was discarded and the cells gently resuspended and incubated for a second 30 min at 4°C with FITC-conjugated polyvalent $F(ab')_2$ sheep anti-human Ig (NBL, Stockholm, Sweden) at a final concentration of 1:6. This second reagent had been absorbed at 1:10 with 50:50 mixture ORBCγ and ORBCμ at 4°C for 1 hr to remove cross-reactivity to rabbit antibodies, and spun at 20,000 r.p.m. for 30 min prior to use to remove aggregates. After this incubation, the Tγ rosettes were washed twice in RPMI 1640 as described above, gently resuspended in a 100-μl volume, and fluorescent rosettes scored under epi-illumination as was described above for SIg-positive cells. None of the Tγ rosettes were stained with FITC–$F(ab')_2$ sheep anti-Hu Ig alone.

RESULTS

Comparison of total T cells and T cell subsets in different stages of MS

Table 1 shows the proportions of T cells and T cell subsets in 11 different patients during an acute attack of MS. The PBL of these patients contained low proportions of total T cells and high proportions of Tγ and Tμ cells compared with normal peripheral blood. They also had a 3-fold greater proportion of Tγ rosettes that stained positively for FcRμ (Tγμ cells) in the PBL fraction compared to controls. While Tγ cells did not increase in number after overnight incubation, the proportion of Tμ cells in PBL doubled. There was a higher proportion of T cells in the CSF lymphocytes than in the PBL from the same patient and than in control PBL. Tγ cells represented twice as many T cells in CSF as they did in PBL (61% *vs* 30%) while Tμ cells formed approximately the same percentage of T cells in both CSF and PBL. Tγμ cells represented 2·5-fold more of the Tγ cells in CSF than in PBL of acute MS patients. The proportion of Tμ cells in fresh CSF (day 0) was 80% of the Tμ population assessed after overnight incubation.

Table 2 shows the results of those chronic patients with active disease at a time usually several months after an attack. CSF total T cells were only slightly higher than PBL T cells and both were lower than the normal control value. MS PBL Tγ and Tμ cells were higher than those in control PBL, and CSF Tγ cells were higher than PBL Tγ cells in the same patient, results similar to those

Table 1. Acute MS patients. Proportions of T cells and T cell subsets in PBL and CSF

MS patients (sex/age)		Lymphocytes ($\%T_{total}$)	T_{total} %Tγ		%Tμ		Tγ (%Tγμ)	Controls (sex/age)		Lymphocytes ($\%T_{total}$)	T_{total} %Tγ		%Tμ		Tγ (%Tγμ)
		Day 0	0	1	0	1	0			Day 0	0	1	0	1	0
1. F/27	PBL	75	35	35	24	83	15	1. F/32	PBL	79	27	26	22	63	9
	CSF	85	44	38	50	73	—								
2. F/34	PBL	54	30	23	50	73	—	2. F/30	PBL	79	24	21	35	49	—
	CSF	26	60	63	30	78	—								
3. F/43	PBL	74	56	57	44	67	20	3. F/40	PBL	78	22	20	19	61	8
	CSF	94	100	73	73	62	38								
4. F/50								4. None							
	CSF	95	81	—	67	—	21								
5. F/23	PBL	66	32	20	47	86	8	5. F/20	PBL	63	13	9	35	82	2
	CSF	91	53	77	48	71	47								
6. F/26	PBL	—	48	—	40	—	24	6. F/22	PBL	—	17	—	57	—	6
	CSF	—	83	—	96	—	62								
7. M/50	PBL	50	25	15	17	61	6	7. M/52	PBL	67	15	7	29	70	2
	CSF	94	50	56	65	74	50								
8. F/39	PBL	60	32	—	25	—	18	8. F/37	PBL	75	23	—	31	—	9
	CSF	94	36	—	50	—	25								
9. F/55	PBL	55	27	—	40	—	19	9. F/65	PBL	70	17	—	31	—	9
	CSF	80	60	—	50	—	50								
10. F/38	PBL	70	30	—	21	—	16	10. F/33	PBL	78	22	—	24	—	6
	CSF	83	70	—	40	—	45								
11. F/30	PBL	60	59	—	55	—	37	11. F/37	PBL	69	33	—	48	—	21
	CSF	78	68	—	60	—	48								
Mean ± s.e.m.															
	PBL	63±3	37±4	30±7	36±4	74±5	18±3		PBL	73±2	21±2	17±4	33±4	65±5	6±1
	CSF	82±7	64±6	61±7	57±5	72±3	43±4								

Table 2. Chronic MS patients. Proportions of T cells and T cell subsets in PBL and CSF

MS patients (sex/age)			Lymphocytes (% T_{total})	T_{total} %Tγ		T_{total} %Tμ		Tγ (%Tγμ)
			Day 0	0	1	0	1	0
1.	F/23	PBL	61	15	17	15	25	6
		CSF	72	88	43	63	44	25
2.	M/38	PBL	67	17	18	20	49	10
		CSF	66	53	65	45	19	15
3.	M/46	PBL	60	29	26	40	61	47
		CSF	73	67	82	52	68	45
4.	M/46	PBL	78	47	49	42	58	—
		CSF	66	61	73	11	58	69
5.	F/31	PBL	71	26	35	45	87	—
		CSF	66	69	77	82	91	—
6.	F/45	PBL	68	33	—	28	—	10
		CSF	69	80	—	80	—	47
7.	M/43	PBL	60	26	—	38	—	14
		CSF	95	58	—	50	—	29
Mean ± s.e.m.								
		PBL	66±3	28±4	29±6	33±4	56±10	17±8
		CSF	72±4	68±5	68±7	55±9	56±12	38±8

Controls (sex/age)			Lymphocytes (% T_{total})	T_{total} %Tγ		T_{total} %Tμ		Tγ (%Tγμ)
			Day 0	0	1	0	1	0
1.	F/26	PBL	82	13	15	38	40	3
2.	M/34	PBL	66	30	22	9	29	12
3.	M/44	PBL	74	15	18	23	36	20
4.	M/45	PBL	83	29	30	26	47	—
5.	F/28	PBL	88	10	29	52	85	—
6.	F/39	PBL	75	17	—	31	—	3
7.	M/42	PBL	78	17	—	21	—	6
		PBL	78±3	19±3	23±3	29±5	47±10	9±3

Table 3. Stable MS patients. Proportions of T cells and T cell subsets in PBL and CSF

MS patients (sex/age)			Lymphocytes (%T_{total})	T_{total} %$T\gamma$		%$T\mu$		$T\gamma$ (%$T\gamma\mu$)
			Day 0	0	1	0	1	0
1.	F/55	PBL	71	17	15	26	60	—
		CSF	70	53	—	20	—	—
2.	F/58	PBL	74	38	38	43	68	35
		CSF	77	67	69	66	83	29
3.	M/35	PBL	—	35	50	20	50	27
		CSF	—	51	—	88	—	30
4.	F/54	PBL	50	28	19	36	52	18
		CSF	92	50	29	89	67	25
5.	F/50	PBL	77	39	—	16	—	20
		CSF	91	58	—	60	—	62
6.	M/39	PBL	77	24	—	34	—	21
		CSF	90	67	—	68	—	62
Mean ±s.e.m.		PBL	70±5	30±4	31±8	29±4	58±4	24±3
		CSF	84±4	58±3	49±20	65±10	75±8	42±8

Controls (sex/age)			Lymphocytes (%T_{total})	T_{total} %$T\gamma$		%$T\mu$		$T\gamma$ (%$T\gamma\mu$)
			Day 0	0	1	0	1	0
1.	F/60	PBL	77	22	14	18	36	—
2.	F/57	PBL	71	26	26	40	42	14
3.	M/31	PBL	—	17	26	39	40	14
4.	F/58	PBL	68	22	19	17	57	7
5.	F/49	PBL	64	30	—	13	—	13
6.	M/35	PBL	74	32	—	56	—	13
		PBL	71±2	25±2	21±3	31±7	44±5	12±1

Table 4. Summary. Proportions of T cells and T cell subsets in PBL and CSF

Total MS patients		Lymphocytes ($\%T_{total}$)	T_{total} %Tγ		T_{total} %Tμ		Tγ (%Tγμ)
	Day	0	0	1	0	1	0
Mean ± s.e.m.							
	PBL	66±2	33±2	30±4	33±3	63±4	20±2
	CSF	79±3	64±3	62±5	58±4	66±5	41±4

Total controls		Lymphocytes ($\%T_{total}$)	T_{total} %Tγ		T_{total} %Tμ		Tγ (%Tγμ)
	Day	0	0	1	0	1	0
	PBL	74±1	21±1	20±2	31±3	54±5	9±1

in the acute disease stage (Table 1). $T\gamma\mu$ cells were also elevated in the chronic stage compared to controls.

Table 3 shows the results of stable patients. Similar to the acute stage, but differing from the chronic stage, the total T cells in CSF was significantly greater than the total T cells in PBL of these patients. $T\gamma$ cells, $T\mu$ cells and $T\gamma\mu$ cells in stable CSF formed a greater proportion of T cells than in PBL total T cells; these values were also higher than controls.

Compared to acute and stable patients, chronic patients in recovery periods seemed to have depressed total T cells and $T\mu$ cells in their CSF.

Tables 1, 2 and 3 also show that the sum of per cent $T\gamma$ plus per cent $T\mu$ in CSF of all disease stages was greater than 100%, an indirect proof of the presence of $T\gamma\mu$ cells bearing FcR for both IgG and IgM. There did not appear to be a correlation of the proportion of T cells in CSF with stage of disease.

Pooled data from all MS patients in Tables 1–3 are summarized in Table 4. In all tables, $T\gamma\mu$ proportions have been expressed as percentages of $T\gamma$ cells. Multiplying this percentage by the per cent $T\gamma$ cells of total T cells shows that by immunofluorescence, 2% of all T cells in control PBL are $T\gamma\mu$, while 6% of T cells in MS PBL are $T\gamma\mu$, and 20–30% of T cells in MS CSF are $T\gamma\mu$.

DISCUSSION

In this paper we have examined subsets of T cells in the CSF of patients with MS and have compared these to PBL of the same patients and PBL of age- and sex-matched controls. As previously reported (Kam-Hansen *et al.*, 1978; Sandberg-Wollheim & Turesson, 1975), we have found a higher proportion of total T cells in CSF lymphocytes of MS patients than in PBL of the same patients and higher than the control PBL T cells (Tables 1–4). With respect to the fluctuations of total T cells in the CSF during different phases of disease when compared with acute patients, we found a depression in CSF T cells in chronic patients during remission to a percentage equivalent to that in PBL. This finding is in agreement with Traugott (1978) but in disagreement with Kam-Hansen *et al.* (1978) who found total CSF T cells higher during remission than exacerbation. Furthermore, stable patients, having had no attack in 12 months or more, resembled acute patients with higher total T cells than chronic patients, results different from Levinson *et al.* (1976) who found lower total CSF T cells in stable patients compared to acute patients. The difference in our results and the above two reports might be partially explained by our having used AET-treated SRBC to detect even the low-affinity E rosetting T cells and our having subdivided patients in remission into two groups—chronic with active disease and stable with inactive disease.

We report for the first time on the proportions of T cell subsets in CSF of MS patients as these are defined by their FcR (Ferrarini *et al.*, 1975; Moretta *et al.*, 1975). As we previously reported for T cells in MS PBL (Merrill *et al.*, 1980), there exists a subset of T cells in MS CSF bearing FcR for both IgG and IgM ($T\gamma\mu$), representing an even larger proportion of total T cells in the CSF (20–30%) than in PBL (6%, see Results). These were detected both by (1) the indirect observation that the sum of per cent $T\gamma$ plus per cent $T\mu$ was greater than 100%, suggesting doubles and (2) the direct observation using the double-marker system.

While the appearance of maximum numbers of $T\mu$ cells in PBL seems to require an overnight incubation in tissue culture (Tables 1–4; Moretta *et al.*, 1975; Golightly & Golub, 1979; Pichler, Lum & Broder, 1978), it is of interest that freshly prepared day-0 CSF T cells contain 80–90% of the $T\mu$ cells seen after overnight incubation (day 1) (Tables 1–4). Because there is an extremely low amount of IgM synthesized within the CSF (Link, 1978), $T\mu$ cells' $FcR\mu$ may not be blocked or modulated to an undetectably low density as has been the postulated reason for low $T\mu$ cell numbers in freshly isolated PBL (Moretta *et al.*, 1975; Bolhuis & Nooyen, 1977). Alternatively, the $FcR\mu$ on CSF T cells may perhaps have a particularly low turnover rate and are less labile than $FcR\mu$ on PBL T cells (Fanger & Lydyard, 1979; Romagnani *et al.*, 1979). Because of the fact that day-0 expression of $FcR\mu$ was nearly maximal and, in many cases, there were too few CSF T cells to culture overnight, we have reported $T\gamma\mu$ proportions for day 0 only, as we feel these values are

an accurate reflection of the double FcR-positive cells as determined by the fluorescence method, though this method may underestimate the number of $T\gamma\mu$ cells (Merrill *et al.*, 1980).

It has been shown that exposing $T\gamma$ cells to IgG-containing immune complexes causes a modulation of the FcR (Moretta, Mingari & Romanzi, 1978; Pichler *et al.*, 1978). While Moretta *et al.* (1978) claim that the loss of $FcR\gamma$ is irreversible, Pichler *et al.* (1978) have observed that some T cells can resynthesize their $FcR\gamma$ after exposure to immune complexes and incubation at 37°C overnight. Furthermore, some $T\gamma$ cells after further incubation synthesize FcR for IgM in addition to resynthesizing $FcR\gamma$ or switch entirely to $T\mu$ cells. They have suggested that modulation of $FcR\gamma$ by IC activates some T cells, driving them through a maturational transition population $T\gamma\mu$ on the way to becoming $T\mu$ cells. This would imply that the high proportions of $T\gamma\mu$ cells in freshly drawn MS PBL and CSF that we have observed, prior to exposure to IC, might be the result of *in vivo* activation by immune complexes. This is a possibility since immune complexes have been described in MS PBL (Goust *et al.*, 1978) and CSF (Levy & Schoen, 1977). *In vivo* activation of CSF lymphocytes is further suggested by high spontaneous proliferation of CSF cells in mitogen-stimulation assays (Kam-Hansen, Frydén & Link, 1979) and in response to myelin basic protein (Lisak & Zweiman, 1977).

On the other hand, a $T\gamma\mu$ cell could be a stem cell giving rise to either a $T\gamma$ cell or a $T\mu$ cell. Preliminary studies on the functional characteristics of the subset suggest that these T cells are active in IgG-dependent ADCC but do not mediate NK killing. Furthermore, they appear to be distinguishable from $T\gamma$ and $T\mu$ cells in their response to mitogens. Further characterization is in progress to determine the place of a $T\gamma\mu$ cell in the T cell lineage in terms of maturation and function to determine if the presence of a large proportion of these cells may play some role in the continuing MS disease.

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