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The distribution of interleukin-2 receptor bearing lymphocytes in multiple sclerosis: evidence for a key role of activated lymphocytes

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SUMMARY

The identification of T cells in the brain using monoclonal antibodies has suggested a role for T cells in the pathogenesis of multiple sclerosis (MS). In the present study the monoclonal antibody anti-Tac, shown to react with interleukin-2 (IL-2) receptors expressed on activated T cells, was used to determine levels of recently activated T cells in blood, cerebrospinal fluid (CSF) and brain sections from MS patients at different stages of disease.

The CSF of MS patients contained much higher numbers of IL-2 receptor positive lymphocytes (up to 67%) than blood cells from the same patients, or the CSF of patients with non-inflammatory neurological diseases. In histological sections of the brain of MS patients with active disease, perivascular lymphocytes expressing IL-2 receptors were detected, as were lymphocytes containing IL-2. In contrast, these were absent in brain sections from patients with chronic MS, secondary demyelination or from normal controls. These observations in CSF and brain suggest that in multiple sclerosis, T-cell activation is occurring within the CNS and not in peripheral lymphoid tissue.

Keywords Multiple sclerosis activated T lymphocytes IL-2 receptors

INTRODUCTION

Multiple sclerosis (MS) is a demyelinating disease of the human central nervous system (CNS), with highly variable periods of relapse and remission. Histologically, areas of primary demyelination are found with perivascular inflammatory cells close to or within acute lesions.

While there is controversy concerning the role of T-cell mediated immunity in the pathogenesis of MS, there is evidence of multiple abnormalities in the immune status of MS patients (reviewed by Waksman, 1981). T cells have been found to be the major cell type present in perivascular cuffs (Nyland *et al.*, 1982; Traugott, *et al.*, 1983), and these lymphocytes may be involved in a T-cell mediated immune reaction against an antigen or virus in the brain. However, it has not yet been demonstrated that the T lymphocytes are functionally active at the sites where demyelination occurs, neither is there agreement about the role of antigen-sensitized cells in the pathogenesis of MS. The availability of the monoclonal antibody anti-Tac (Uchiyama *et al.*, 1981a,b) which is directed against the interleukin-2 (IL-2) receptor, has provided a convenient index of recent T-cell

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activation, as its expression fades within 7–10 days after antigenic stimulation (Cantrell & Smith, 1983). This antibody offers a major advantage over other activation markers, such as HLA-DR (Ia) antigens, because it is readily detected only on activated T cells, and not on B cells or macrophages, although there are recent indications of low levels of IL-2 receptor expression on activated B cells (Tsudo *et al.*, 1984).

The relevance of studies on circulating peripheral blood lymphocytes (PBL) from patients with localized diseases or delayed-type hypersensitivity is questionable (Fox *et al.*, 1982; Adamson *et al.*, 1983; Platt *et al.*, 1983). However, further insight into the pathogenesis of MS may be gained by studies of lymphocytes isolated from cerebrospinal fluid (CSF) which have recently trafficked through the CNS rather than PBL.

In addition, it is known that the cells involved in an active immune response localize around the antigen, and thus in MS may be sequestered in and around the plaque. To investigate this possibility, we have used anti-Tac to compare the levels of activated T cells in the blood, CSF and brain of patients with MS and other neurological diseases (OND). Similar techniques have been used to show T-cell activation in sections of lung from sarcoid patients (Semenzato *et al.*, 1984).

METHODS

Patients. MS patients were classified as in relapse when a new clinical sign had developed within the last 3 weeks; patients with active disease had a clear previous history of new signs or symptoms within the last 3 months. Patients with stable disease had no new sign or symptom for 6 months or more. Patients were classified as progressive cases with evidence of lesions at two or more separate sites in the CNS and also a history of progressive paraplegia. All MS patients were identified by the criteria of McDonald & Halliday (1977). None of the patients or controls were on immunosuppressive therapy. Whenever possible, blood samples were taken at the same time as CSF was obtained.

Cells. Peripheral blood lymphoid cells (PBL) were obtained from whole heparinized blood, diluted with an equal volume of RPMI 1640 (Gibco Europe, Paisley, UK), and centrifuged over Ficoll-Hypaque (Pharmacia, Hounslow, UK) at 1000*g* for 15 min. After washing, cells were resuspended at $1–2 \times 10^6$/ml in RPMI 1640 medium supplemented with 5% new-born bovine serum (Flow Laboratories, Irvine, UK) containing 0·2% sodium azide. CSF lymphocytes were concentrated by centrifugation at 400*g* for 10 min, washed twice in serum-free RPMI 1640, and resuspended to a final volume of approximately 75 μl.

Immunofluorescent staining of lymphocytes. $1–2 \times 10^5$ PBL were incubated at 4°C with 50 μl of murine anti-Tac monoclonal antibody (generously donated by Dr T. Waldmann, NIH) or UCHT1 (anti-T3) (generously donated by Dr P. C. L. Beverley, London) for 30 min. After two washes with cold medium, 50 μl of fluorescein-conjugated rabbit anti-mouse IgG (Nordic Immunological Laboratories, Maidenhead, UK) was added for 30 min at 4°C. Cells were washed and resuspended in 1 ml medium and analysed for fluorescence on a Fluorescence Activated Cell Sorter (FACS IV, Becton Dickinson UK Ltd), at University College Hospital. Control experiments showed no staining of four Epstein-Barr virus (EBV) lines at the concentration of anti-Tac used in this study (1 μg/ml).

25 μl aliquots of CSF lymphocytes (between 5×10^3 and 10×10^3) were incubated for at least 2 h at 37°C on poly-L-lysine-coated 13-mm coverslips. Cells were washed for 30 min with RPMI 1640 medium containing 5% new-born bovine serum, before addition of 50 μl of monoclonal antibodies for 30 min at 4°C. Coverslips were washed three times, and 50 μl of Rhodamine-conjugated rat anti-mouse IgG (donated by Dr F. Walsh, Institute of Neurology, London) were added for 30 min, over ice. The final washes of CSF lymphocytes were with serum-containing (three times) then serum-free medium (twice). Coverslips were mounted, and viewed by eye using a Leitz Dialux 20 EB fluorescent microscope, with at least 50 cells per slide being counted.

Brain tissue. A total of nine MS brains were studied from which 14 blocks of tissue were examined. These were obtained by courtesy of Professor I. Allen (Royal Victoria Hospital, Belfast), Dr M. Esiri (Radcliffe Infirmary, Oxford), Dr W. Tortellotte (National Neurologic Research Bank, Los Angeles) and Dr M. L. Cuzner (Institute of Neurology, London). In addition two normal

brains and one case of carbon monoxide-induced demyelination were used as controls. 10 μm cryostat sections were cut from frozen blocks of tissue and stored at $-70°C$.

ABC immunoperoxidase staining of brain tissue sections. Frozen sections were left at room temperature for 30 min and then fixed in acetone for 8 min (or 90% ethanol for 2 min in the case of anti IL-2 antibody (3·9C2)). In addition to anti-Tac and UCHT1 (see above), two other monoclonal antibodies were used. Firstly, DA-2 (anti-Ia) (generously donated by Dr J. Bodmer, Imperial Cancer Research Fund, London) and secondly, 3·9C2 (generously donated by Dr C. Lewis, Glaxo Group Research, Middlesex) which is specific for IL-2. Control experiments using this antibody showed negligible staining of a mouse myeloma, an EBV line and human peripheral blood lymphocytes. However, the anti-IL-2 antibody does appear to cross react with some fibrillar component of human brain and this is currently under investigation. Reagents from the 'Vectastain' ABC Kit (mouse IgG) (Vector Laboratories, Inc., California) were used and diluted in phosphate buffered saline (PBS). PBS was used for all washes between incubations. Sections were treated with 2% normal horse serum and then with the primary antibody both for 30 min. Biotinylated horse anti-mouse IgG (0·05%) was applied for 30 min, and then slides were incubated for 45 min with a mixture of 1% Avidin DH and 1% biotinylated horseradish peroxidase H. The reaction product was visualized by a 5 min incubation of 0·05% diaminobenzidine in 0·05 M Tris buffer (pH 7·2) containing 0·01% H_2O_2. After a wash in water the sections were counterstained with haematoxylin blue.

Statistics. Mean percentages of Tac^+ cells from different patient groups were compared using Student's *t*-test.

RESULTS

In the first part of this study the proportion of Tac^+ T-lymphocytes was determined in the blood of healthy controls and of patients with MS and OND.

The percentages of peripheral blood T cells labelled with monoclonal antibody (anti-Tac) were determined using the Fluorescence Activated Cell Sorter and in a few cases by counting fields under light microscopy. As expected in healthy controls the proportion of Tac^+ cells was very low and in many cases undetectable (Fig. 1) as reported by others (Platt *et al.*, 1983). Similar low values were found for some of the patients with other neurological diseases but in two patients with inflammatory disease (e.g. post-operative infection) markedly increased proportions were found. In patients with MS there was a small but significant increase in the proportion of Tac^+ cells ($P < 0·05$) in comparison with healthy cases. However, no clear correlation was found in the proportion of Tac^+ cells with the disease status of the MS patients. Close to a relapse either low or high proportions of Tac^+ cells were seen in different individuals.

These findings contrasted with the proportion of Tac^+ lymphocytes recovered from the CSF (Fig 2). Considerable numbers of Tac^+ CSF cells were observed, and in some cases of MS more than half the total T lymphocytes expressed the Tac antigen (mean $= 22·0 \pm 4·1\%$). In only 7/26 (27%) samples was the proportion of Tac^+ cells less than 5%. Marginal pleocytosis was found in relatively few CSF samples from MS cases, with only 7/26 (27%) MS samples having CSF white blood cell (WBC) counts greater than 5/mm^3. However, none of these samples contained any polymorphonuclear leukocytes. Although no definite correlation between WBC counts and percentage Tac^+ cells in the CSF was observed, CSF from the patient with the highest cell count (13/mm^3) also showed the highest proportion of Tac^+ cells (67%). As a comparison, none of these MS patients' levels of Tac^+ cells in peripheral blood exceeded 4% (mean $= 1·1 \pm 0·2\%$) (Fig. 3). In CSF from OND patients, high proportions of Tac^+ cells were only evident in inflammatory diseases and, unlike the MS patients, polymorphonuclear cells were also present (Table 1). Even in these cases the proportion of Tac^+ cell in peripheral blood did not exceed 4%. However, CSF from OND patients showing no signs of inflammatory CNS disease (i.e. low CSF WBC counts and only a small number of polymorphs) contained negligible numbers of Tac^+ cells (see Table 1), making the Tac levels in CSF samples from MS patients highly significant ($P < 0·001$).

Figure 2 shows the numbers of Tac^+ cells in CSF of active, stable and progressive MS patients.

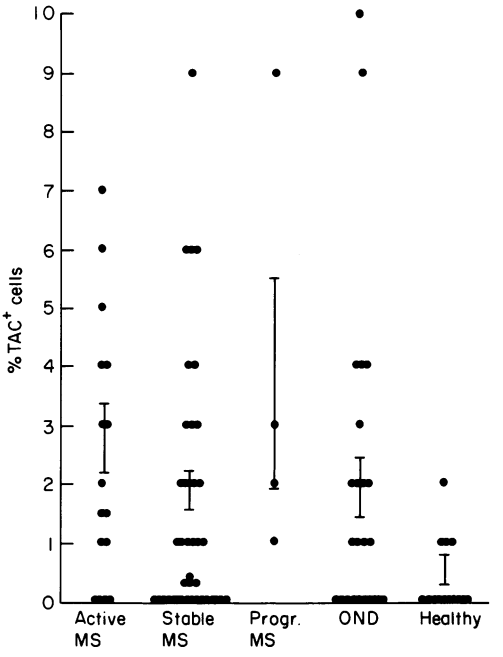

Fig. 1. Percentage of Tac^+ lymphocytes in peripheral blood from MS, OND and healthy individuals. Progr = progressive. Each point represents the value for an individual patient. Error bars show mean ± s.e. Values are: active MS: 2·8 ± 0·8%, stable MS: 1·9 ± 0·3%; progressive MS: 3·8 ± 1·8; OND = 1·9 ± 0·6%; healthy: 0·4 ± 0·1%.

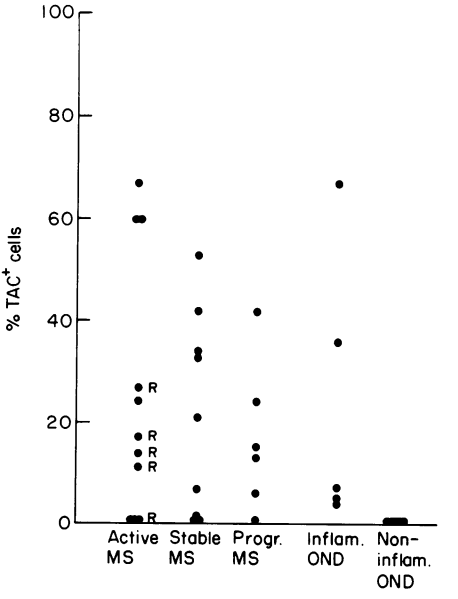

Fig. 2. Percentage of Tac^+ lymphocytes in CSF from MS and OND patients. Progr = progressive; Inflam = inflammatory; R = relapse. Values for mean ± s.e. are: active MS: 25·5 ± 7·7%; stable MS: 21·2 ± 6·4%; progressive MS: 16·7 ± 6·1%; inflammatory OND: 20·6 ± 10·6%; non-inflammatory OND: 0 ± 0%.

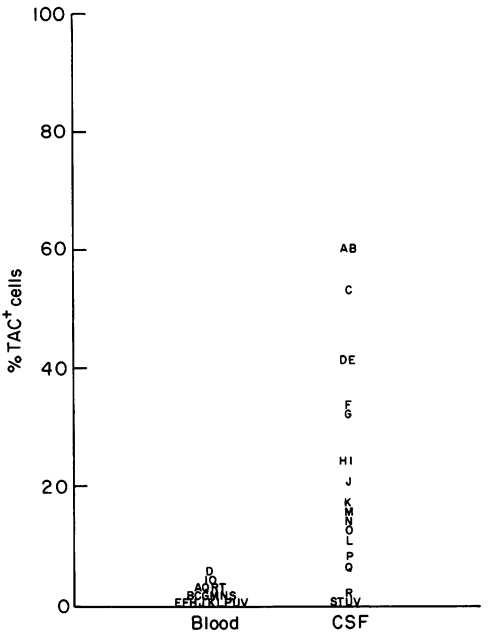

Fig. 3. Comparison of Tac$^+$ levels in CSF and blood of MS and OND patients. Each letter represents an individual patient.

Table 1. Interleukin-2 receptor positive T-lymphocytes in blood and CSF of patients with other neurological diseases

Diagnosis	Polymorphonuclear cells (% total cells)	CSF WBC counts/mm^3	Tac$^+$ cells (%) CSF	Tac$^+$ cells (%) PB
Craniotomy*	(i) 30	20	67	0
	(ii) 3	9	5	ND
Acoustic neuroma*	10	22	36	4
Foramen magnum decompression*	37	83	7	4
Foramen magnum decompression*	50	690	4·5	3
Foramen magnum decompression*	67	500	4	1
Sensory neuropathy	0	3	0	ND
Secondary demyelination	0	2	0	ND
Craniotomy	2	16	0	ND
Secondary demyelination	0	1	0	ND
Benign intracranial hypertension	0	1	0	ND

*=CNS infection
ND=Not done

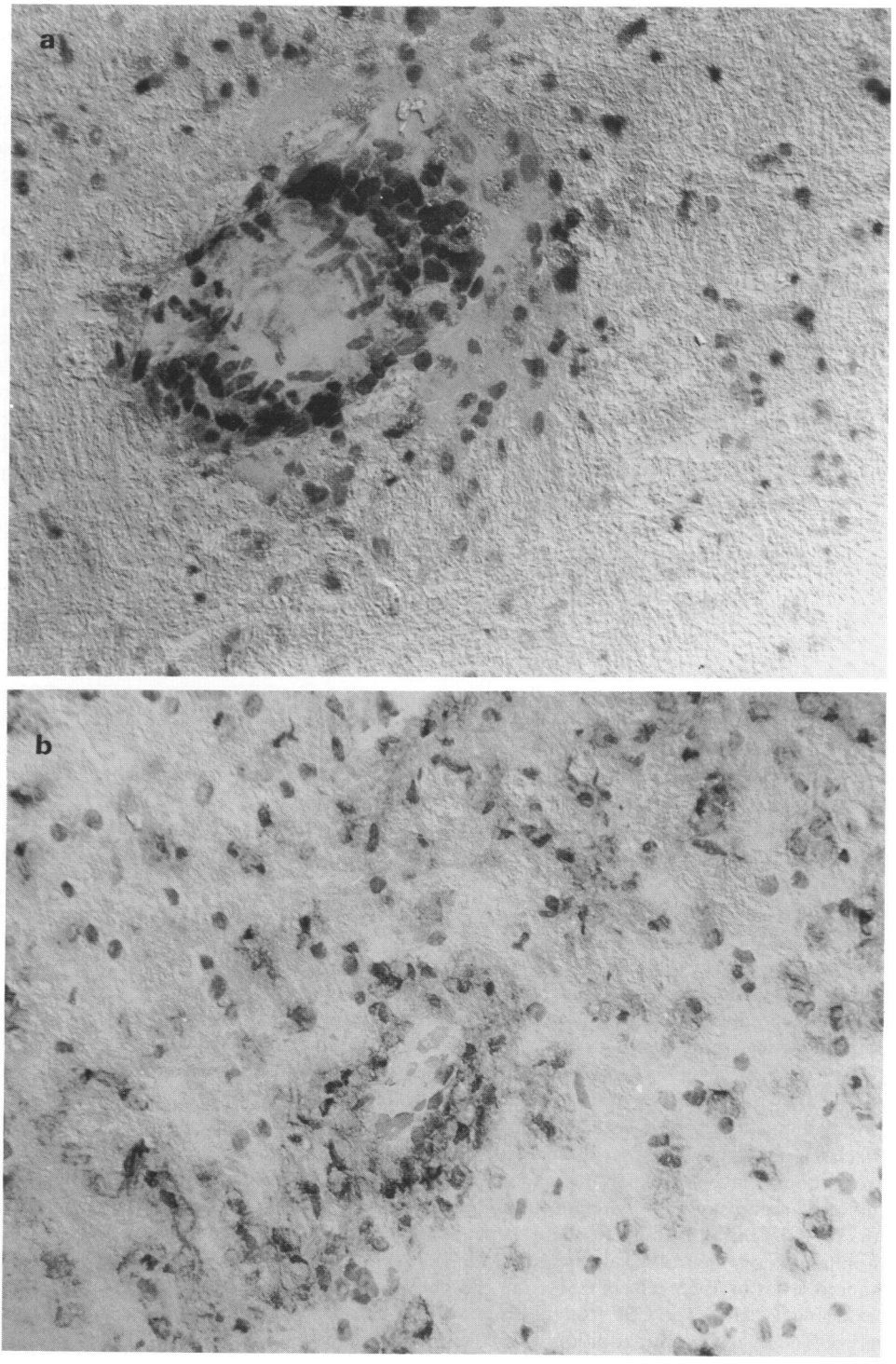

Fig. 4. Demonstration of (a) Tac^+ and (b) $DA\text{-}2^+$ lymphocytes in cryostat sections of active multiple sclerosis lesions by an Avidin-biotin immunoperoxidase technique ($\times 250$).

The mean percentage of Tac^+ cells was higher in the active group ($25{\cdot}5 \pm 7{\cdot}7$) compared to that for either stable ($21{\cdot}2 \pm 6{\cdot}7$) or progressive disease ($16{\cdot}7 \pm 6{\cdot}1$). This difference, however, was not statistically significant ($P > 0{\cdot}05$).

It is interesting to note that five of the CSF samples were obtained from MS patients within 3 weeks of a relapse and contained 27%, 17%, 14%, 11% and 0% Tac^+ lymphocytes respectively. These levels were not significantly higher than those from CSF samples taken during other disease stages. This implies, despite the small sample size that, as in the peripheral blood, there is no direct relationship between disease stage and the presence of activated cells.

These results could be readily explained if activation of T cells occurred at the site of inflammatory reaction within the CNS. Therefore, we examined cell populations in perivascular cuffs close to or in active lesions in MS brains. Immunoperoxidase labelling of serial sections with UCHT1 confirmed the previous observation that the majority of the cells in perivascular infiltrations in MS are T cells (Nyland *et al.*, 1982; Traugott *et al.*, 1983). Of 11 active lesions studied (from 6 cases), 2 of these (from a single case) showed the presence of Tac^+ cells (Fig. 4a). In addition, cells with IL-2 localized on their membrane and within the cytoplasm ($3{\cdot}9C2^+$) were found in greater numbers in these two lesions than Tac^+ cells, but no $3{\cdot}9C2^+$ lymphocytes were identified in the other active sections. All the active sections studied contained many DA-2^+ (MHC Class II, HLA-DR or Ia^+) cells, both within perivascular cuffs and in the paranchyma (Fig. 4b), as reported by Traugott *et al.* (1983). Staining of sections from either a case of secondary demyelination, two normal brains or two chronic MS cases revealed a negligible number of UCHT1^+ and DA-2^+ cells and no Tac^+ or 3-$9C2^+$ cells.

DISCUSSION

We have found that there is only a small but significant increase in the proportion of Tac^+ PBL in MS patients compared to healthy controls or those with non-infectious disease. No correlation has been found between disease activity and the frequency of Tac-bearing lymphocytes in blood. This may be because, as indicated in adoptive transfer experiments of chromium labelled myelin basic protein-sensitized rat lymphocytes, sensitized cells only stay in the circulation for a few days before sequestration in brain and lymphoid tissue (Naparstek *et al.*, 1983). Thus it was found that human volunteers injected with tetanus toxoid only had detectable Tac^+ cells in blood for 24 h (Yachie *et al.*, 1983). Similarly in subjects with delayed-type hypersensitivity to tuberculin (Platt *et al.*, 1983), lymphocytic infiltration in Sjogren's syndrome (Adamson *et al.*, 1983) and rheumatoid arthritis (Fox *et al.*, 1982), activated T cells were found at the local site, although these were not detectable in the blood.

In the CSF, evidence, for DNA synthesis in lymphocytes has been obtained by analysis with a Fluorescence Activated Cell Sorter and autoradiography. Noronha *et al.* (1980) and Reunanen (1982) found selective effects on CSF lymphocytes from MS patients, with either the number of S-phase cells or the labelling index being increased respectively. In addition, Dommasch *et al.* (1977) and Yamakawa *et al.* (1976) observed increased labelling indices with increased disease activity.

Activation antigens have been detected in the CSF lymphocyte population. Data from Marrosu *et al.* (1983) implied the presence of $T3^+/Ia^+$ cells in the CSF (i.e. activated T cells) but they did not perform double labelling experiments to confirm this suggestion. The results reported here show significant amounts of Tac^+ lymphocytes to be present in the CSF from MS patients compared to both Tac^+ levels of PBL from these same patients (Fig. 3), and levels in CSF from other non-inflammatory neurological diseases (Fig. 2). There appears to be an underlying trend of enhanced levels of Tac^+ cells in CSF from MS patients with active disease compared with stable disease (Fig. 2). However, CSF from patients within 3 weeks of a relapse did not show the highest levels of Tac^+ cells and, in addition, CSF from some patients with active disease (i.e. within 3 months of a relapse) contained negligible levels of Tac^+ cells. These discrepancies may be explained by the transient expression of IL-2 receptors (Cantrell & Smith, 1983) as well as factors determining the accumulation and efflux of Tac^+ cells in the CSF during the disease, such as the distance between the site of activation and entry into the CSF, and the variable relationship between

inflammation and neurological signs or symptoms. The finding of activated cells in the CSF of MS patients not only in acute exacerbations, but also in progressive and stable disease agrees with the study by Noronha *et al.* (1980, 1982), who found proliferating $T8^-$ cells in CSF from patients during all phases of MS, and implies that an ongoing immunological response is an integral part of the MS disease process.

Several OND control patients were studied and their levels of Tac^+ cells in CSF fell into two groups, depending on the presence of CNS inflammation as judged by levels of polymorphonuclear cells in the CSF (Fig. 2). Only those CSF samples with significant numbers of polymorphs contained any Tac^+ lymphocytes. Hence, in the absence of clinical or laboratory signs of inflammatory disorder, the presence of Tac^+ cells in CSF may be an indication of MS. This observation is in agreement with Noronha *et al.* (1980) who found no proliferating cells in CSF samples from OND patients, when polymorphonuclear cells were absent.

In order to determine the source and possible site of activation of the CSF cells, brain sections were examined by an immunoperoxidase method. Immunohistochemical techniques have been used to study the composition of the mononuclear infiltrate characteristic of MS brain plaques (see also Booss *et al.*, 1983; Traugott *et al.*, 1983). The presence of Tac^+ and $3.9C2^+$ cells in the perivascular cuffs of two active MS brains indicates that localized T-cell activation can occur under certain conditions (Fig. 4a). However, this would not appear to happen in all active lesions since 9/11 sections did not contain Tac^+ or $3.9C2^+$ cells, despite the presence of infiltrating T lymphocytes and Ia-bearing cells. A likely explanation of this observation could be related to the fact that T-cell activation results in only transient expression of the Tac antigen (Cantrell & Smith, 1983) for less than 2 weeks. Hence, activated T cells may be present during the initiation of a plaque but not at the later disease stages usually found in post-mortem material. The presence of significant numbers of Ia^+ cells in sections from active cases of MS suggests a role of antigen presentation in this disease and is similar to the Ia-staining observed in tissues from known autoimmune diseases, such as thyroiditis (discussed Bottazzo *et al.*, 1983).

Since there are much higher proportions (0–67%) of Tac^+ cells in the CSF of MS patients compared to the blood (0–9%), it is concluded that cellular activation occurs centrally rather than peripherally. In addition, the many Tac^- lesions suggest that T-cell activation occurs early in the disease process, with the activated cells perhaps subsequently migrating to the CSF. The central activation of lymphocytes may be a factor involved in the failure of systemic immunosuppressive drugs in the treatment of MS. Further work is in progress to analyse the mechanism of CNS lymphocyte activation.

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