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Immunology

Colocalization of lymphocytes bearing γδ T-cell receptor and heat shock protein hsp65$^+$ oligodendrocytes in multiple sclerosis

KRZYSZTOF SELMAJ*, CELIA F. BROSNAN, AND CEDRIC S. RAINE

Departments of Pathology (Neuropathology) and Neuroscience, and the Rose F. Kennedy Center for Research in Mental Retardation and Human Development, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461

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ABSTRACT **The presence of T lymphocytes bearing the γδ T-cell receptor (TCRγδ) has been studied immunocytochemically in central nervous system (CNS) tissue from 13 patients with multiple sclerosis (MS), 4 with inflammatory non-MS CNS disorders, 6 with other neurological diseases, and 3 with nonneurologic conditions. Twenty-eight of 43 MS lesions contained TCRγδ cells and these were most frequently found in chronically demyelinated areas, in contrast to the previously studied CD4$^+$, CD8$^+$ (TCRαβ) population, which predominated in more active lesions. Some TCRγδ lymphocytes had an unusual morphology with long dendritic processes, which were sometimes interconnected forming a network. Because it is generally believed that TCRγδ lymphocytes function in a cytotoxic fashion in association with heat shock proteins (hsp), we examined the colocalization of TCRγδ cells with 65- and 70-kDa hsp (hsp65 and hsp70) in MS lesions. Hsp65 was expressed on immature oligodendrocytes at the margins of chronic lesions containing TCRγδ lymphocytes. The coexpression of these molecules might imply functional relationships perhaps of significance to the chronicity of the MS disease process and the failure of CNS remyelination.**

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the human central nervous system (CNS). The presence of lymphocytes and macrophages within CNS lesions implicates an immunologic basis to the disease process, although the antigen responsible remains to be determined. The nature of the lymphocytic response, with respect to the expression of CD4 and CD8 molecules, has been extensively studied in both the CNS and peripheral blood (1, 2). More recently, efforts have been directed toward the analysis of T-cell receptor (TCR) α- and β-chain rearrangements and growing evidence indicates a restricted pattern of TCRαβ cells associated with the disease (3, 4). To date, however, no reports on MS have addressed the potential role of T cells expressing the TCRγδ.

T cells expressing the TCRαβ and -γδ are thought to represent distinct cell lineages and rearrangement of these two sets of genes probably occurs independently (5). T lymphocytes bearing the γδ receptor usually lack expression of CD4 and, on rare occasions, express CD8. In normal individuals, they comprise a minor population of T lymphocytes (6). The organization of both the γ and δ chains has been established and the germ-line-encoded repertoire has been shown to be limited (7). Diversity of the TCR appears to be achieved by junctional variation (8). Two distinct constant (C) region gene segments ($C_\gamma1$ and $C_\gamma2$) are used by TCRγδ cells. The $C_\gamma1$ can form interchain disulfide bonds, whereas the $C_\gamma2$ occurs in a non-disulfide-linked form (9). The monoclonal antibody (mAb) δ-TCS1 recognizes $V_\delta1$–$J_\delta1$ and $V_\delta1$–$J_\delta2$ gene rearrangements (V, variable; J, joining), which are often (but not always) associated with $C_\gamma2$-encoded non-disulfide-linked TCR (9, 10) gene product and the mAb TCRδ1 is thought to recognize all TCRγδ cells. The biological function of TCRγδ cells remains to be established but evidence has been provided that these cells are involved in the response to antigens, including heat shock proteins (hsp), which are expressed during tissue damage (11). TCRγδ cells might in this way initiate or amplify autoimmunity. In the present study, we have assessed the occurrence and distribution of TCRγδ cells in MS lesions of different ages, and the frequency of TCRγδ cells in the peripheral blood of MS patients and normal controls has been presented in abstract form elsewhere (12). The findings have revealed an unusual association of TCRγ lymphocytes with long-standing CNS lesions in MS in which 65-kDa hsp (hsp65) was coexpressed on immature oligodendrocytes. This association introduces a new concept underlying the chronicity of the MS lesion and the failure of the CNS to remyelinate extensively.

MATERIALS AND METHODS

Tissue Samples. Early postmortem material (<6 hr) was obtained from 13 MS patients. From each, two or three optimal cutting temperature compound (Miles)-embedded blocks from CNS plaques and adjacent white matter were studied. Three groups of controls were used: 3 CNS samples from patients dying from nonneurological diseases with no indication of CNS involvement, 6 samples from patients who died from noninflammatory CNS disease (4 Alzheimer, 1 Parkinson, and 1 multisystem degeneration), and 4 CNS inflammatory non-MS conditions [2 tropical spastic paraparesis (TSP), 1 subacute sclerosing panencephalitis (SSPE), and 1 adrenoleukodystrophy (ALD)]. Frozen blocks of spleen tissue from most patients were studied for control purposes.

Immunocytochemistry. Frozen sections were stained with an avidin–biotin–peroxidase complex (ABC) technique. These were air-dried, fixed in acetone (10 min at 4°C), quenched for peroxidatic activity with 0.03% hydrogen peroxide, and blocked with normal serum. They were then incubated with primary antibody overnight at 4°C, washed, and incubated with biotinylated horse anti-mouse IgG or goat anti-rabbit IgG for 60 min at room temperature. The ABC reagent was applied for 60 min and diaminobenzidine was used as the chromogen. Sections were dehydrated and mounted in Permount and some were counterstained with hematoxylin.

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Abbreviations: MS, multiple sclerosis; TCR, T-cell receptor; CNS, central nervous system; LT, lymphotoxin; TNF, tumor necrosis factor; mAb, monoclonal antibody; C, constant; V, variable; J, joining; MBP, myelin basic protein; GC, galactocerebroside; MAG, myelin-associated glycoprotein; GFAP, glial fibrillary basic protein; PBL, peripheral blood lymphocyte; IL, interleukin; hsp, heat shock protein; TSP, tropical spastic paraparesis; SSPE, subacute sclerosing panencephalitis; ALD, adrenoleukodystrophy.

*To whom reprint requests should be addressed.

Antibodies. The following mAbs were used: anti-human CD3 (1:100; Becton Dickinson); TCRδ1 (13, 14), and δ-TCS1 (15) raised against the human δ chains of the TCR, and βF1 (16) raised against the human β chain (all used at 1:10; T Cell Sciences, Cambridge, MA); anti-human tumor necrosis factor (TNF) (1:50; Olympus, Lake Success, NY); and anti-human lymphotoxin (LT) (1:2; Boehringer Mannheim). Polyclonal antibody to LT and TNF was also tested (1:100; Genzyme). To distinguish immune cells from CNS elements, serial sections were stained with the following antibodies: rabbit anti-bovine myelin basic protein (MBP; 1:100) and (anti-galactocerebroside (GC; 1:200) (both generated in this laboratory); anti-myelin-associated glycoprotein (MAG; 1:100) (gift of Norman Latov, Columbia University, New York); anti-Leu 7 (1:100) (clone HNK-1; Becton Dickinson); anti-glial fibrillary acidic protein (GFAP; 1:200) (gift of James Goldman, Columbia University); anti-LeuM-5 (1:5) (Becton Dickinson); anti-neurofilament protein (1:100) (gift of Peter Davies, Albert Einstein College of Medicine). For hsp localization, anti-HeLa hsp72 (Amersham), and anti-*Mycobacterium leprae* hsp65 (clones Y1.2 and Y1.5) (gift of Vijay Mehra, Albert Einstein College of Medicine) were used (17). The latter antibodies recognize the highly conserved N-terminal region of the protein and show cross-reactivity with other hsp65 (18).

Patients and Mononuclear Cell Isolates. Fourteen MS patients, 12 chronic progressive and 2 with remitting–relapsing disease, were used for fluorescence-activated cell sorter (FACS) analysis. All patients were clinically active at the time of study and fulfilled the diagnostic criteria for MS, including magnetic resonance imaging (19). Eight healthy controls of age and sex similar to the MS patients were assayed. Peripheral blood mononuclear cells were isolated with LeucoPrep cell separation tubes (Becton Dickinson).

Immunofluorescent Staining and FACS Analysis. Mononuclear cells from MS patients and control subjects were stained by indirect immunofluorescence. Briefly, 1×10^6 cells per sample were incubated with primary unlabeled mAb for 60 min, followed by goat anti-mouse IgG (1:20) conjugated with fluorescein for another 60 min, washed twice in phosphate-buffered saline, and analyzed in a FACS II (Becton Dickinson).

RESULTS

TCRγδ Cells in MS Lesions and Correlation with Lesion Activity. TCRδ1$^+$ cells were present in 28 of 43 MS lesions (9 of 13 MS patients). To define further the distribution of these cells, MS lesions were classified into three categories: acute (evolving lesion, indistinct edge, perivascular and parenchymal infiltration, hypertrophic astrocytes, little gliosis), chronic active (established lesion, well demarcated edge, perivascular infiltration at edge, intensely gliotic), and chronic silent (same as chronic active but with little or no cellular infiltration), according to accepted criteria (20).

Ten plaques were classified as acute and three of these were positive for TCRδ1$^+$ cells. However, the majority of lymphocytes associated with the lesion were CD3$^+$ TCRδ1$^-$ (Fig. 1). In these lesions, TCRδ1$^+$ cells were commonly detected at the lesion edge, with some infiltration into adjacent white matter (Table 1). They rarely occurred in perivascular cuffs. In contrast, CD3$^+$ TCRδ1$^-$ cells occurred throughout the lesion but were primarily located perivascularly. When assessed as a component of CD3$^+$ cells, TCRγδ cells comprised a minor constituent (Fig. 1).

Twelve plaques were classified as chronic active. In these, TCRδ1$^+$ cells were identified in 8 of 12 lesions (Table 1) with numbers that ranged from a minor to a major component of the CD3$^+$ cells present. In contrast to acute lesions, all chronic active lesions demonstrated TCRδ1$^+$ cells predominantly within the lesion center and to a lesser extent at the lesion edge (5 of 8 lesions) and in adjacent white matter (3 of 8 lesions) (Table 1). As noted in acute plaques, TCRδ1$^+$ cells were rarely observed in perivascular cuffs.

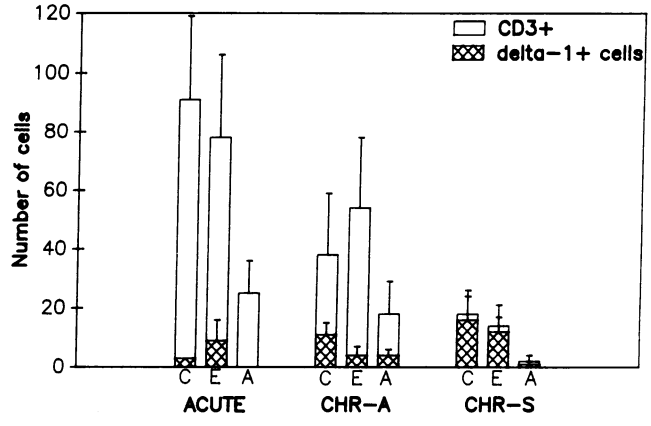

FIG. 1. Distribution and relative proportion of δ1$^+$ cells. Open bars, mean number of CD3$^+$ cells in specificed areas of MS lesions (C, center; E, edge; A, adjacent white matter) per 0.07 mm^2; cross-hatched bars, mean number of TCRδ1$^+$ cells in the same lesion areas.

Twenty-one plaques were classified as predominantly chronic silent and CD3$^+$ cells were relatively rare. TCRδ1$^+$ cells were found with greatest frequency in this type of lesion (17 of 21) (Table 1). In these plaques, 16 of 17 (94%) showed TCRγδ cells in the lesion center, 14 of 17 (82%) showed TCRγδ cells at the lesion edge, and 7 of 17 (41%) showed TCRγδ cells in adjacent white matter (Table 1). Strikingly, TCRδ1$^+$ cells were the predominant CD3$^+$ lymphocyte (Fig. 1). As described above, TCRδ1$^+$ cells were rarely found in perivascular cuffs, being located primarily in the parenchyma.

Of the TCRδ1$^+$ cells in MS brain tissue, <15% were positive for δ-TCS1, suggesting that the majority of these cells expressed the disulfide form of the TCRγδ receptor.

In three groups of control CNS tissue including four inflammatory conditions, TCRδ1$^+$ cells were not prominent. Among these non-MS inflammatory controls, two cases of TSP were studied intensively. While TCRαβ cells were readily detectable in both cases, one contained no TCRγδ T cells, and the other contained only an occasional TCRγδ cell.

Morphology of TCRγδ Cells in MS Lesions. The TCRδ1$^+$ cells found in MS plaques presented with two types of morphology. The first was lymphocyte-like, consisting of rounded cells staining similarly to lymphocytes detected with other anti-lymphocyte antibodies (Fig. 2). The second had a dendritic morphology. A notable feature of these cells was the presence of uni- or bipolar processes with adhesion plates frequently present at the terminal end of each filopodium (Fig. 3). These attenuated processes often connected with adjacent TCRγδ cells, creating a network of TCRδ1$^+$ cells extending into adjacent white matter.

Because of the dendritic morphology of the TCRγδ cells and their superficial similarity to CNS elements, studies were

Table 1. Presence of TCRδ1$^+$ cells in lesions at different stages of activity

Type of lesion	No. of TCR lesions	Lesion area		
		Center	Edge	AWM
Active	3/10	1/3	3/3	0/3
Chronic active	8/12	8/8	5/8	3/8
Chronic silent	17/21	16/17	14/17	7/17

Data are expressed as ratio of the number of positive lesions to the total number of lesions analyzed. AWM, adjacent white matter.

performed on serial sections stained with a variety of anti-CNS antibodies to exclude the possibility that the cells were of CNS origin. The results showed that these $CD3^+$ cells did not stain with anti-MBP, anti-MAG, anti-GC, or anti-Leu7 (therefore excluding oligodendroglial origin); anti-GFAP (therefore not astrocytes); anti-LeuM-5 (not microglia); and anti-neurofilament antibodies (not neuronal). The dendritic $TCR\delta1^+$ cells in MS lesions rarely showed reactivity with δ-TCS1.

Hsp Expression in MS Lesions. Since it has been suggested that $TCR\gamma\delta$ cells respond to hsp, we stained MS brain tissue with mAbs raised against the *M. leprae* hsp65 and the HeLa hsp72. The *M. leprae* protein shares homology with another hsp65 (17). Anti-HeLa hsp72 showed no specificity for the MS lesion. However, in the diffusely myelinated edge of the chronic lesions, identified in hematoxylin and eosin-stained sections, we were able to detect hsp65 on large cells (Fig. 4). Serial sections of the same MS lesions showed the presence of $TCR\delta1^+$ cells. Unaffected CNS tissue from MS brains did not show immunoreactivity for the 65-kDa protein. Also, in four controls (two TSP, one ALD, and one SSPE), no reactivity to hsp65 was detected. Serial sections of MS lesions stained with a variety of anti-CNS antibodies (see above) suggested that the 65-kDa protein was expressed by reactive oligodendrocytes at lesion margins in diffusely myelinated (remyelinated?) white matter. Staining with anti-MBP revealed intense reactivity by large cells with the same distribution as the anti-$hsp65^+$ cells but in greater numbers (Fig. 5). These cells corresponded to reactive cells of oligodendroglial lineage. Adjacent normal white matter stained less intensely with anti-MBP.

$TCR\gamma\delta$ Cells in MS and Normal Spleen. The occurrence of $TCR\delta1^+$ cells was compared in normal spleens with spleens obtained from MS patients. In normal spleen, $TCR\delta1^+$ cells accounted for 10–15% of $CD3^+$ cells and the same proportion of these cells was found in MS spleens. Some $TCR\delta1^+$ cells showed a dendritic morphology similar to that found in MS brain (Fig. 6), but these dendritic $TCR\delta1^+$ cells occurred in similar numbers in spleens from normal and MS patients. It has also been reported (6) that $TCR\delta1^+$ cells in normal spleen belong primarily to the $\delta\text{-}TCS1^+$ phenotype. In the tissue used in this study, <20% of these cells were $\delta\text{-}TCS1^+$ in both normal and MS spleen.

Characterization of Cytokine Production. Since $TCR\gamma\delta$ cells express potent cytolytic activity (21, 22) and produce several cytokines including TNF (22), we stained serial MS sections with mAbs and polyclonal antibodies against human TNF and human LT. The results showed that no $CD3^+$ cells stained for TNF. Some cells with lymphocyte-like morphology stained for LT but these cells were located exclusively in a perivascular location and serial sections revealed that they corresponded to $CD3^+$, $\beta F1^+$ cells, linking them to the $TCR\alpha\beta$ lymphocyte population.

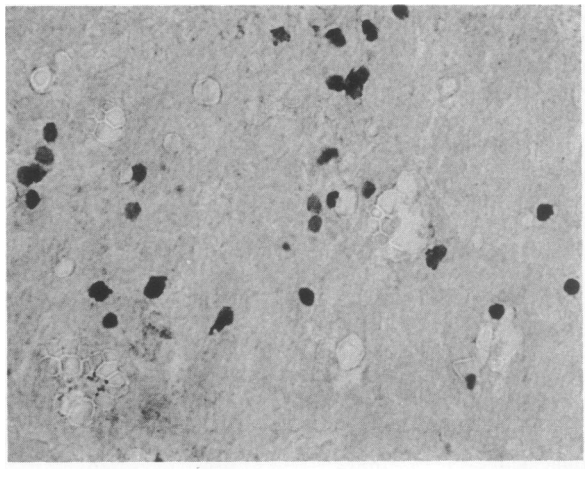

FIG. 2. Immunohistochemical demonstration of $TCR\delta1^+$ cells with a lymphocyte-like morphology in the center of a chronic active lesion. (×290.)

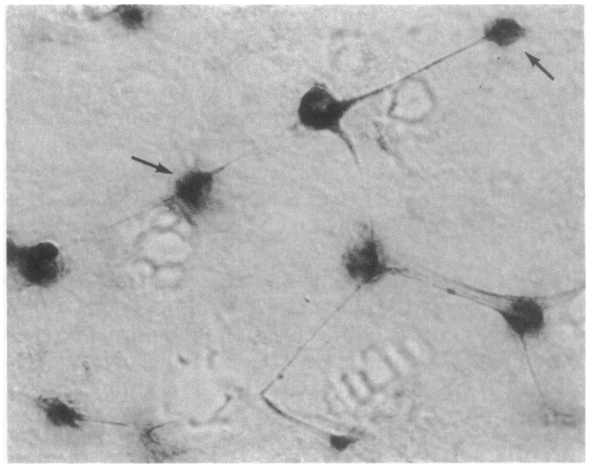

FIG. 3. $TCR\delta1^+$ cells in the center of a chronic silent lesion. A filopodium with a terminal adhesion plaque is shown (arrows). (×460.)

$TCR\gamma\delta$ Cells in Peripheral Blood. The mean percentage of $TCR\gamma\delta$ cells in MS blood was insignificantly higher than in normal controls and was within the normal range. However, within the MS group, three patients had a higher percentage of $TCR\delta1^+$ cells (14.3%, 9.0%, 10.1%), but no correlation with disease activity was noted. In three patients (two with a higher percentage of $TCR\delta1^+$ cells), $\delta\text{-}TCS1^+$ cells were the

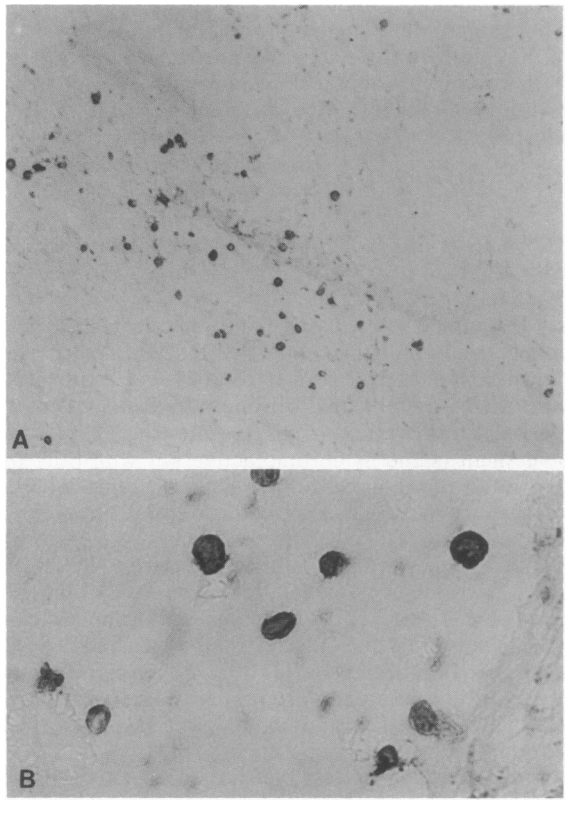

FIG. 4. Same lesion margin as in Fig. 3, stained with anti-hsp65. Note the large $hsp65^+$ cells within the lesion area. (*A*, ×110; *B*, ×460.)

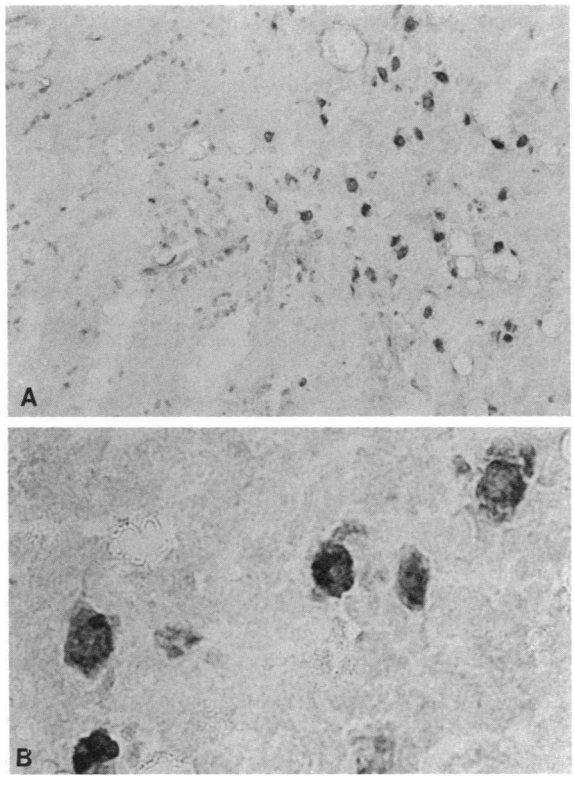

FIG. 5. Same lesion as in Fig. 3, stained with anti-MBP. Note that the MBP$^+$ cells are similar to the hsp65$^+$ cells (Fig. 4) but are more numerous. (*A*, ×110; *B*, ×460.)

major subpopulation. In the remainder, δ-TCS1$^+$ cells were not present or comprised <30% of the TCRδ1$^+$ cells, thus falling into the normal range.

Since all 14 MS patients studied for TCRδ1$^+$ cells in peripheral blood lymphocytes (PBLs) were assessed as clinically active, the results suggested that TCRδ1$^+$ cells did not increase in number in PBLs in association with disease activity. However, because we found in brain tissue that TCRδ1$^+$ cells were present more frequently and in higher number in older, less-active MS lesions rather than in acute lesions, we assessed the occurrence of TCRδ1$^+$ cells in PBLs with relation to disease duration and extent of disability. A higher proportion of TCRδ1$^+$ cells was not found in PBLs in patients with a longer history of MS or in patients with a higher score on the Kurztke disability scale.

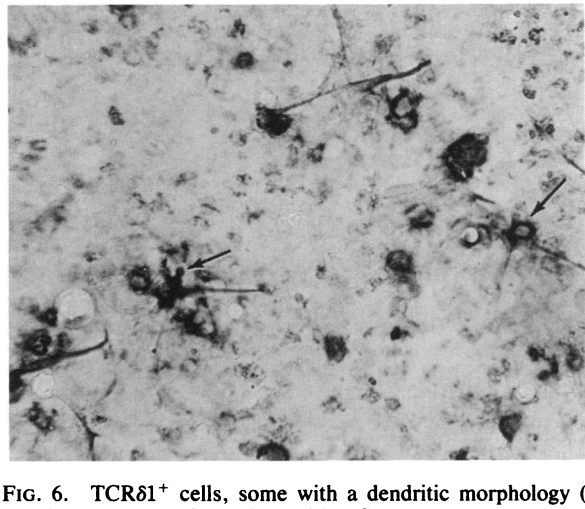

FIG. 6. TCRδ1$^+$ cells, some with a dendritic morphology (arrows), in the spleen of a patient with MS. (×460.)

DISCUSSION

In the present study, we report that TCRγδ cells exist in the CNS of MS patients and are associated with chronic lesions. CNS tissue from 12 control samples (4 non-MS inflammatory, 6 other neurological diseases, 3 normal) showed no staining in TCRγδ cells except for one case of TSP that showed the occasional TCRγδ T cell while TCRαβ cells were always present. TCRγδ cells have been identified at low levels in a number of lymphoid (23) and nonlymphoid (6) tissues, with some predilection for spleen and gut epithelium (24). TCRγδ cells have been identified in greater numbers in some inflammatory conditions (25, 26). To date, this newly recognized subpopulation of lymphocytes has not been reported in the CNS, although two TCRγ clones have been derived from cerebrospinal fluid of SSPE (27). Differences in the distribution of human TCRγδ cells expressing two predominant combinations of V regions $V_\gamma1$–$C_\gamma2$/$V_\delta1$–C_δ and $V\gamma2$–$C_\gamma1$/$V_\delta2$–C_δ have been demonstrated in a variety of tissues. The former, recognized by δ-TCS1, was predominantly located in thymus and spleen, whereas the latter, disulfide form, recognized by the mAbs BB3 or TiγA, predominated in peripheral blood (28). The latter phenotype corresponded to the cells detected in this study.

One of the most interesting findings was related to the morphology of TCRγδ cells in MS brain tissue. Apart from being present as typical lymphocyte-like cells, unique dendritic TCRδ1$^+$ cells predominated in many lesions. TCRγδ cells with an identical morphology, with extended filopodia, have been cloned from the peripheral blood of normal subjects (29). These dendritic cells of the δ-TCS1 phenotype were adherent, spread, and showed active motility in culture. However, in the MS lesions described here, dendritic TCRγδ cells did not express the δ-TCS1 phenotype, suggesting their derivation from cells by rearrangement of their $V_\delta2$ region. In this regard, dendritic TCRγδ cells in MS brains resembled BB3$^+$ dendritic cells identified in interfollicular areas of human tonsillar tissue (6). It remains to be shown whether the dendritic TCRγδ cells in MS plaques represented a distinct functional subpopulation and whether their common occurrence in some MS brains was a CNS-related phenomenon or was pathogenetically linked to the disease. In this regard, while TCRαβ cells were not difficult to detect, we were unable to detect TCRδ1$^+$ cells in brain tissue from three groups of controls, including four inflammatory conditions, except for one of the two cases of TSP that showed an occasional TCRγδ cell in the spinal cord. This may suggest that the chronic MS lesion involves a unique immunopathologic reaction between TCRγδ cells and hsp on oligodendrocytes. However, the specificity of these associations for MS will require examination of a broader spectrum of inflammatory conditions.

The antigen reactivity of TCRγδ cells and their possible role in the immune response is the subject of intense investigation. The limited diversity of the V_γ and V_δ, repertoire suggests that TCRγδ cells might have a specific immune function. It has recently been demonstrated that some TCRγδ clones respond to hsp65 (30). Hsp form a group of highly conserved proteins expressed by both prokaryotic and eukaryotic cells in response to different types of stress, including heat. Heat shock proteins combine several subclasses—ubiquitin, hsp60, GroEL-related proteins, hsp70, and hsp90 (31). Mycobacterial hsp65 shares >50% sequence homology with human cell hsp65 and GroEL-related proteins (18, 32). TCRγδ lymphocytes can be preferentially enriched and proliferate in heterogeneous cell populations exposed to heat shock or cocultured with heat-shocked syngeneic cells (33). Differences in the expression of V_γ and V_δ regions from purified protein derivative-stimulated and heat-shocked TCRγδ cells suggest the existence of TCRγδ cells reactive to

self hsp65. In the present report, we have demonstrated that hsp65 was expressed within demyelinated lesions in MS. Reactivity to self hsp could lead to the initiation or amplification of an autoimmune reaction at the site of tissue injury. TCR$\gamma\delta$ clones reactive with hsp64 have been isolated from synovial fluid of rheumatoid arthritis patients (34). Additional evidence for autoreactivity by TCR$\gamma\delta$ cells has been provided by analyzing TCR$\gamma\delta$ surface-positive hybridomas (35). The spontaneous production of interleukin 2 (IL-2) resulting from cellular activation via the TCR$\gamma\delta$ receptor was a common feature of these hybridomas.

To what extent TCR$\gamma\delta$ lymphocytes responding to hsp might contribute to the demyelinating process in MS is not known. TCR$\gamma\delta$ cells have been shown to lyse several tumor cell lines (21, 22). It has been shown that human purified $CD4^-$, $CD8^-$, $TCR\delta1^+$ cells and clones derived from them proliferated and displayed strong cytotoxicity toward allogeneic cells (36). Several cytokines can be produced by TCR$\gamma\delta$ cells—e.g., IL-4, granulocyte–macrophage colony-stimulating factor, IL-2, and TNF (22). No data are available on TCR$\gamma\delta$ cell interactions with CNS elements but since TNF and LT have been shown to mediate degradation of myelin (37) and are cytotoxic for oligodendrocytes *in vitro* (38, 39), we tested whether TCR$\gamma\delta$ cells in MS brains reacted positively to these cytokines. Although we, and others, were able to identify both TNF and LT in MS tissue (40, 41), there was no overlap with TCR$\gamma\delta$ staining. However, other cytotoxic factors, perforins, and serine esterases, have been associated with TCR$\gamma\delta$ cells (42, 43).

Unexpectedly we found that TCR$\gamma\delta$ cells were infrequent in very active MS lesions in which T cells of $\alpha\beta$ lineage predominated and were more numerous in less active lesions. In the present study, we found that hsp65 was present on large, reactive, proliferating oligodendrocytes. These oligodendrocytes showed intense staining for MBP, an antigen that is expressed at lower levels by mature oligodendrocytes (44). Thus, expression of high levels of MBP by these oligodendrocytes suggested that they were recently derived. Immature oligodendrocytes in demyelinating areas are thought to represent proliferating cells engaged in remyelination (45). The present observation might add hsp65 to the list of markers for remyelinating oligodendrocytes (46, 47). Coexpression of $hsp65^+$ and TCR$\gamma\delta$ in the same lesions implied that immature oligodendrocytes might be the target for TCR$\gamma\delta$ lymphocytes and might help explain why remyelination never proceeds to complete repair in MS. These observations, plus the pattern of distribution of TCR$\gamma\delta$ cells within the lesion, suggested that TCR$\gamma\delta$ cells might not be associated with lesion initiation but might contribute to the persistence of the pathologic process.

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