

OLIGOCLONAL T LYMPHOCYTES IN THE CEREBROSPINAL FLUID OF PATIENTS WITH MULTIPLE SCLEROSIS

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Multiple sclerosis (MS)¹ is a chronic, inflammatory, demyelinating disease of the central nervous system (CNS) that has been postulated to be a T cell-mediated autoimmune disorder (1, 2). At sites of active demyelination, the majority of infiltrating cells are T cells and macrophages, with smaller numbers of B cells (1). In the cerebrospinal fluid (CSF), >85% of cells are T lymphocytes (3). The Igs in both the brain and CSF of MS patients can be electrophoretically resolved into a few bands (oligoclonal banding) and are thought to be synthesized within the CNS by an oligoclonal population of B cells (4, 5). The antigenic specificity of the T cells in the CSF compartment in subjects with MS is unknown.

Two possibilities exist concerning the population of T cells that are present in the CSF of MS patients: (a) the T cells have multiple antigenic specificities and are derived from a large number of progenitor cells (polyclonal T cells); or (b) there are only a few specificities, derived from a small number of progenitor T cells (oligoclonal T cells) or from a single common ancestral cell (clonal T cells). Clonal T cell expansion has been observed in T cell malignancies (6) but not in a nonmalignant inflammatory disease.

Whether two T cells are in fact identical and derived from a common ancestral cell can be determined by analysis of their TCR gene rearrangements using Southern blotting techniques (7, 8). Identical clones contain identically rearranged TCR genes and have identically sized restriction fragments after enzyme digest of DNA and hybridization with different TCR gene probes. To determine by Southern blot analysis whether different CSF-derived T cells are related to one another, it is necessary to expand the original T cell population without unintentional growth of some T cells and not others. Others have demonstrated the clonogenic potential of most every human peripheral blood T cell by grow-

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Abbreviations used in this paper: CNS, central nervous system; CSF, cerebrospinal fluid; HZV, herpes zoster virus; MNC, mononuclear cells; MS, multiple sclerosis; SSPE, subacute sclerosing panencephalitis; TCGF, T cell growth factor.

ing them in vitro in the presence of PHA, irradiated autologous mononuclear "feeder" cells, and IL-2 (9). Using an identical methodology, we have been able to clone individual T cells from the CSF with high efficiency (10).

In the present study, T cell populations were examined by cloning individual CSF and peripheral blood T cells before other in vitro manipulation. The T cells were compared with one another by analyzing the pattern of T cell rearrangements in each clone to examine whether clonal T cell expansion can occur in immune compartments in nonmalignant inflammatory diseases.

Materials and Methods

Patient Population. The characteristics of the five patients studied are presented in Table I. Chronic progressive MS patients (GR and KA) had clinically definite disease and were in the progressive phase as defined by a decline in the previous 9 mo of at least one grade on the Kurtzke disability status scale (11). Patient MU had an acute, fatal, atypical 8-mo course of MS and had received a 12-d, 8-g course of intravenous cyclophosphamide plus intravenous adrenocorticotrophic hormone 2 mo before the lumbar puncture to obtain CSF. Patient WI had subacute sclerosing panencephalitis (SSPE) on the basis of clinical examination and characteristic EEG and spinal fluid examination with high levels of antimeasles antibody (12). Patient VE had herpes zoster virus (HZV) meningoencephalitis (13) that was serologically confirmed by an ELISA analysis of serum and CSF for HZV. The subject with HZV meningoencephalitis had a CSF cell count of 40 lymphocytes/mm³; the other subjects had between 1 and 4 lymphocytes/mm³. Except for subject MU, no patient had received steroids for 1 mo or immunosuppressive medication for 1 yr before the time of blood drawing and lumbar puncture.

Single Cell T Lymphocyte Cloning. Peripheral mononuclear cells (MNC) and CSF MNC were prepared according to standard techniques (10). Peripheral or CSF MNC were diluted and plated at <1 cell/well in 100 μ l of tissue culture medium containing 10⁵/ml irradiated (5,000 rad) autologous, mononuclear feeder cells and purified PHA (Wellcome Research Laboratories, Beckenham, England) at a final concentration of 0.5 μ g/ml in V-bottomed 96-well plates (Flow Laboratories, Inc., McLean, VA) (10). At 48 h, 100 μ l of either 10% T cell growth factor (TCGF) (10) or 5% IL-2 (Advanced Biotechnologies, Silver Spring, MD) was added to each well. Plates were fed every 4 d and samples wells were counted to monitor cell growth. Between days 10 and 14, cells were transferred from V-bottomed wells to round-bottomed 96-well plates with 10⁵ irradiated,

TABLE I
T Cell Clonality in Cerebrospinal Fluid

Subject	Disease	Age	Sex	Duration of disease	Oligoclonal bands	T cell clonality	
						CSF	Blood
		yr					
GR	Chronic progressive MS	34	F	10 yr	Yes	18/28	2/26
KA	Chronic progressive MS	36	F	8 yr	No	5/27	0/6
MU	Acute MS	39	M	8 mo	One band	0/37	0/41
WI	SSPE	16	F	6 mo	Yes	0/33	0/34
VE	HZV	63	M	1 mo	No	0/20	ND
	Meningoencephalitis						
PO	Normal control	39	F	—	—	ND	0/66

* The number of T cell clones demonstrating common sets of unique TCR gene arrangements are shown.

allogeneic mononuclear feeder cells (obtained from a single healthy donor) per well, 0.5 $\mu\text{g/ml}$ PHA, and 10% TCGF or 5% IL-2. Growth-positive wells, ascertained by microscopic analysis, were split, followed 3–4 d later by transfer of cells back into 96-well V-bottomed plates with 5,000 T cells per well and 10^5 irradiated, allogeneic, mononuclear feeder cells, again with PHA and TCGF or IL-2. After 5–10 d of growth, T cell clones were transferred to 25-ml tissue culture flasks (Flow Laboratories, Inc.) at a cell concentration of 10^6 cells/ml. Certain clones have remained stable for >1 yr after restimulation with allogeneic feeder cells, PHA, and IL-2 at 10–14-d intervals. Cloning efficiency of the CSF and blood T cells were similar to previous studies and ranged from 20 to 98% (10).

mAbs and T Cell Phenotyping. Analysis of T cell surface antigens was performed by direct immunofluorescence using fluorescein-conjugated anti-CD4 and anti-CD8 mAbs (Coulter Immunology, Hialeah, FL), followed by flow cytometry on an Epics C analyzer as previously described (3).

Southern Blot Analysis. Genomic DNA extractions were performed according to standard methodology (14). DNA was digested with the restriction enzyme Eco RI or Hind III, size fractionated by electrophoresis through a 0.8% agarose gel, and transferred onto nitrocellulose by the method of Southern (14). Filters were hybridized to nick-translated ^{32}P -labeled probes of the TCR β chain gene and γ chain gene and were washed at 60°C in 0.1% SDS, 0.15 M NaCl, and 0.0015 M Sodium citrate before autoradiography. DNA probes were restriction enzyme DNA fragments of previously cloned germline T cell antigen receptor β chain genes (15). $J_{\beta 1}$ is a 2.6-kb Hind III–Nai I fragment containing the $J_{\beta 1}$ gene segment cluster; $J_{\beta 2}$ are contiguous 1.9-kb Pvu II–Pvu II and 1.5-kb Pvu II–Eco RV fragments containing the 3' half of the $J_{\beta 2}$ gene segment cluster. The two probes do not crosshybridize. J_{γ} is a 0.8-kb Hind III–Eco RI fragment containing $J_{\gamma 1}$. It hybridizes to both the $J_{\gamma 1}$ and $J_{\gamma 2}$ gene segments (8).

Results

The pattern of TCR β chain and γ chain gene rearrangements for each of the clones was determined by Southern blot analysis using DNA probes specific for the $J_{\beta 1}$, $J_{\beta 2}$, and J_{γ} gene segment clusters. A representative set of the Southern blots are shown to demonstrate that these clones can be distinguished from one another (Fig. 1). Of the >250 DNA samples examined, ~5% were observed to have more than two different rearranged fragments hybridized to either the $J_{\beta 1}$ or $J_{\beta 2}$ probe (see clone 37, Figure 1 B). We have assumed that these samples were derived from cell cultures in which more than one T cell was originally cultured; none of these samples were included in the analysis of T cell clonotypes. Furthermore, we found that the amount of hybridization to restriction fragments in the germline configuration varied between DNA samples, (e.g., the 4.4-kb germline $J_{\beta 2}$ Eco RI fragment in Fig. 2). This occurred because of the contribution in each sample of DNA from varying numbers of irradiated, mononuclear feeder cells.

We examined the pattern of β chain and γ chain gene rearrangements of 318 independently derived T cell clones from three subjects with MS, two subjects with other inflammatory diseases of the CNS, and from the blood of a normal subject. The DNA from 28 independent CSF-derived T cell clones from subject GR were digested with the restriction enzyme Eco RI and hybridized in separate experiments with three TCR gene probes: $J_{\beta 1}$, $J_{\beta 2}$, and J_{γ} . The restriction fragment patterns of each clone were compared with the pattern produced by all the other clones to determine if any two clones were identical to one another. Identity of two cloned T cells could not be determined by comparison of restric-

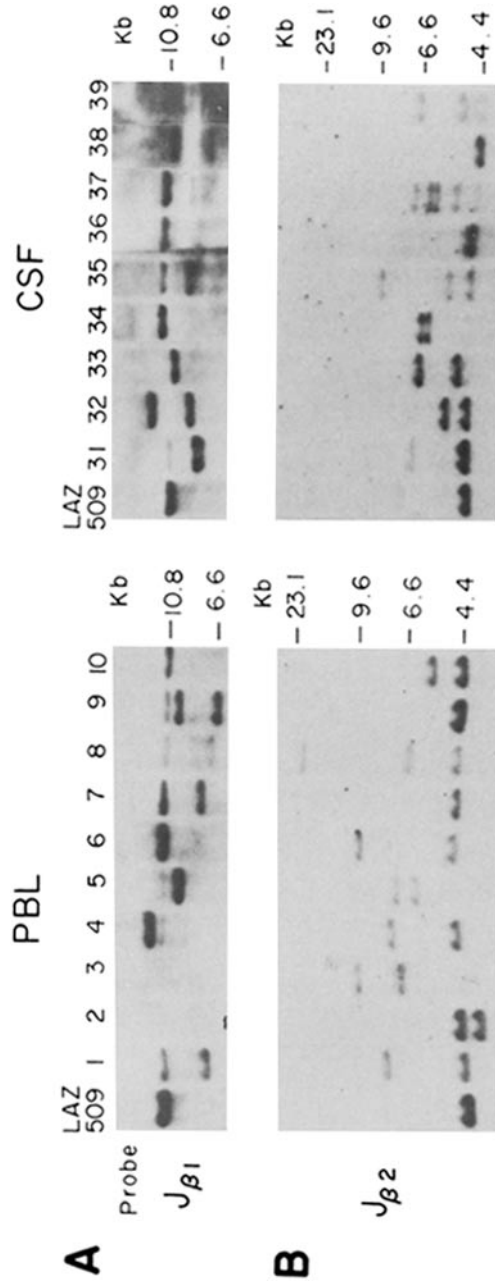


FIGURE 1. Southern blot analysis of β and γ chain gene rearrangements in T cell clones derived from the PBLs and CSF of patient GR with chronic progressive MS. DNA from the B cell line, LAZ 509, and each of the PBL and CSF T cell clones were digested with Eco RI, fractionated on an agarose gel, transferred to nitrocellulose, hybridized to $J_{\beta 1}$ (A) and $J_{\beta 2}$ probes, (B), and analyzed by autoradiography. Southern blot analyses of a representative number of T cell clones is shown. Clones exhibiting more than two rearranged DNA fragments, e.g., CSF clone 37 (B), were not included in the analysis.

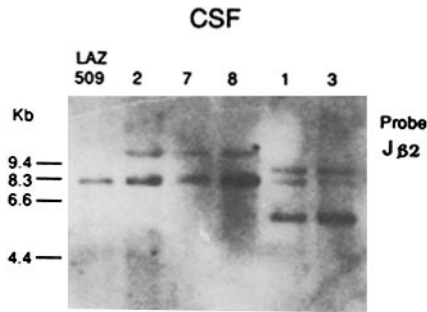


FIGURE 2. Identical pattern of $J_{\beta 2}$ gene segment rearrangement in T cell clones derived from the CSF of patient GR with chronic progressive MS. The identical pattern of two rearranged DNA fragments hybridizing to the $J_{\beta 2}$ probe is shown for 7/8 CSF-derived T cell clones displaying this pattern. These clones have one rearranged DNA fragment hybridizing to the $J_{\beta 1}$ probe as exemplified by clone 33 in Fig. 1 in addition to an identical rearrangement pattern of the J_{γ} gene segment (not shown). Because of the contribution of varying amounts of irradiated, mononuclear feeder cell DNA, the 4.4-kb Eco RI DNA fragment, containing the germline $J_{\beta 2}$ gene segment cluster, appears in varying intensity in the different samples.

tion fragments identified by a single probe. That is, two clones may produce comigrating fragments on the basis of chance. To eliminate this possibility, Southern blots were hybridized with a second independent probe. The possibility that two different clones would produce two comigrating fragments is extremely unlikely. Finally, the use of a third independent probe (J_{γ}), as used in these experiments, should completely eliminate the possibility that two different clones would appear identical by chance alone. Of the 28 CSF clones from subject GR, there were five sets of gene rearrangement patterns identified among more than one clone; each set of unique patterns consisted of rearrangements observed in different experiments with $J_{\beta 1}$, $J_{\beta 2}$, and J_{γ} probes. One set of gene rearrangement patterns identified in different experiments with $J_{\beta 1}$, $J_{\beta 2}$, J_{γ} probes (clone 33, Fig. 1) was found in 8 of the 28 clones studied. This clonotype is shown for seven of the eight clones in Fig. 2. Other sets of common gene rearrangements were observed in the CSF of subject GR, and they are summarized in Table I. Two sets of common rearrangement patterns were each found twice among the 26 independent blood-derived T cell clones from subject GR (Table I). One clonotype was found in one CSF-derived T cell clone and one blood-derived T cell clone (data not shown). All of the clones with common TCR gene rearrangements were $CD4^{+}$, $CD8^{-}$.

27 clones were established from another patient (KA) with chronic progressive MS. One set of common rearrangement patterns using $J_{\beta 1}$, $J_{\beta 2}$, and J_{γ} probes after an Eco RI digest was found among three clones, and another among two clones. Fig. 3 is a Southern blot that shows the identical rearrangement patterns

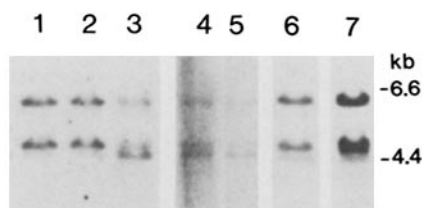


FIGURE 3. Identical patterns of $J_{\beta 2}$ gene segment rearrangements after Hind III enzyme digest in separate T cell clones derived from the CSF of patient KA with chronic progressive MS. In addition, identical patterns of $J_{\beta 1}$, $J_{\beta 2}$, and J_{γ} gene segment rearrangements were observed from CSF T cell clones of subject KA after Eco RI digest and hybridization with $J_{\beta 1}$, $J_{\beta 2}$, and J_{γ} probes (not shown). This was confirmed by an additional digest of genomic DNA with the restriction enzyme Hind III and hybridization to the $J_{\beta 1}$, $J_{\beta 2}$, and J_{γ} probes ($J_{\beta 2}$ shown above).

of T cell clones from subject KA after digestion and hybridization of genomic DNA with an additional restriction enzyme (Hind III) and TCR gene probe ($J_{\beta 2}$) combination.

41 and 37 clones were generated from blood and CSF, respectively, of subject MU with acute, fatal MS. There were no common rearrangement patterns observed among the blood- and CSF-derived T cell clones.

34 blood-derived and 33 CSF-derived T cell clones were generated from a subject with SSPE, 20 clones were generated from the CSF of a subject with HZV meningoencephalitis, and 66 clones were generated from the blood of a normal subject (Table I). There were no common β chain gene rearrangements observed indicating that these were polyclonal T cell populations in the CSF and blood.

Discussion

In these experiments, T cells were directly cloned from the CSF and blood of patients with MS and other inflammatory CNS diseases before other in vitro manipulation and were compared by Southern blot analysis of the rearrangement of their T cell antigen receptor β chain and γ chain genes. We found there was clonal expansion of T cell populations in the CSF of two patients with chronic progressive MS, as compared with the CSF of subjects with other inflammatory CNS disease, a patient with acute, fatal MS, or normal peripheral blood. In one patient with progressive MS, two identical clones were isolated from the peripheral blood.

Southern blotting has a limited ability in differentiating two restriction fragments that have migrated on an agarose gel. Thus, in the type of analysis presented in these experiments, it is important to use more than one restriction enzyme or probe to prove that two clones have identical rearrangements. For these experiments, we used two enzymes and three different DNA probes that identify rearrangements of TCR genes to determine the relationship between different T cell clones. We estimate that these probes allow us to determine if two T cell clones are identical and thus derived from a common progenitor with a very high level of confidence ($p > 0.001$) based on the resolution of agarose gels with Southern blotting and with the use of different probe and restriction enzyme combinations, each of which gives an independent assessment of clonality. In addition, the analysis has been confirmed by the recent identification of the V gene group used by the eight clones shown in Fig. 2, where we found that the same V gene is used (Hafler, D. A., and A. D. Duby, manuscript in preparation).

Clonal T cell expansion has most commonly been demonstrated in T cell malignancies and T cell lymphocytosis with cytopenia (16–19). Oligoclonal T cells have not generally been found in studies examining the T cell response to foreign antigens (20), although certain V-D-J sequences of TCR β chain genes may preferentially be used in the T cell response (21). It is as yet unknown whether the clonally expanded CSF T cell populations represent discrete antigenic specificities against certain antigenic determinants (21). Nonetheless, the analysis presented here demonstrates for the first time to our knowledge that

accumulation and/or expansion of clonal populations of T cells can occur in an immune compartment such as CSF during the course of a nonmalignant inflammatory response.

Attempts to define the specificity of CSF T cells found in MS patients have been unsuccessful (1, 2). In previous experiments (10, 22, 23), we and others have been unable to demonstrate T cell clones derived by single cell cloning from the peripheral blood, CSF, and brain tissue of MS patients that proliferate in response to myelin basic protein or proteolipid protein. The oligoclonal CSF T cells we have identified may reflect pathologic autoreactive clones to an as yet undefined antigen important in the initiation or mediation of demyelination. Alternatively, the oligoclonal population of T cells in the CSF may reflect the chronic nature of the disease process with local proliferation of T cells that play no pathogenic role. Nevertheless, our data indicate that limited specificities may exist among the CSF T cell populations in chronic progressive MS patients, although their clinical importance is as yet unknown.

The oligoclonal T cells that we isolated were $CD4^+$, $CD8^-$. Because $CD4^+$ cells can be either helper inducer, suppressor inducer, or class II MHC antigen-restricted cytotoxic effector cells, it is not possible to ascribe a particular cell function to our T cell clones (24). Whether these clonally expanded T cells are antigen-reactive inducer cells or cytotoxic effector cells is an important question that is presently under investigation.

Oligoclonal Ig banding found in MS CSF remains unexplained (4, 5). The oligoclonal CSF T cells that we observed could be related to, or responsible for, this oligoclonal Ig production, and studies are now in progress to determine whether the oligoclonal T cells are specifically directing the oligoclonal B cell synthesis of CSF antibodies in MS.

The presence of clonal expansion of CSF lymphocytes from the first two chronic progressive MS patients examined, and the absence of this finding in the subject (MU) with acute fatal MS is of interest. It is possible that either the acute nature of his disease or previous immunosuppression may have influenced the frequencies of the CSF T cell population. Alternatively, oligoclonal populations may have been present in acute MS and subjects with the inflammatory CNS diseases but represented too small a fraction of the total T cell population to be detected by this methodology. Of note is a recent study of two MS patients in which oligoclonal T cells were not detected in the CSF (25). Further studies on larger series of patients are necessary to examine whether clonal T cell expansion in MS CSF is a rare or frequent event.

Given the results we have obtained, further analysis of T cells from the CSF of MS patients should also define: (a) whether the appearance of oligoclonal T cells is an early or late event in the evolution of MS; (b) whether there is cross-reactivity between oligoclonal TCR clonotypes from different MS patients; and (c) whether there are common variable region TCR genes found in higher frequencies among different MS patients. The approach described here also has general applications for examining the clonality of T cells in the immune compartments of other inflammatory diseases, such as rheumatoid arthritis, autoimmune thyroiditis, transplant rejection, and insulin-dependent diabetes mellitus. Further examination of expanded TCR clonotypes in immune compartments

and their relationship to antigenic specificities may be of importance in attempting to further understand the cellular immune response in inflammatory human diseases.

Summary

We have investigated the T cell populations in the cerebrospinal fluid (CSF) of chronic progressive multiple sclerosis (MS) patients. Individual T cells from the CSF and blood were cloned before expansion and their clonotypes were defined by analysis of rearranged T cell receptor β chain and γ chain genes. 87 T cell clones from blood and CSF of two patients with chronic progressive MS were examined for common TCR gene rearrangement patterns. In one patient, 18 of 28 CSF-derived T cell clones demonstrated common TCR gene rearrangements indicating oligoclonal T cell populations; in the blood, two patterns were found twice among 26 T cell clones. In another patient, 5 of 27 CSF-derived clones had common TCR gene rearrangement patterns. In contrast, no common β chain rearrangement pattern was found among 67 T cell clones derived from the blood or CSF of a patient with subacute sclerosing panencephalitis, among 20 clones from the CSF of a patient with herpes zoster meningoencephalitis, or among 66 clones from a normal subject. A subject with atypical, fatal MS of 8-mo duration was also studied and did not have oligoclonal T cells in the CSF or blood. These results demonstrate that distinct oligoclonal T cell populations can be found in the CSF immune compartment of subjects with nonmalignant inflammatory disease and they can create a new avenue for the investigation of the specificity of the T cell response within the central nervous system.

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Note added in proof: Similar to our findings in the cerebrospinal fluid, oligoclonal populations of T cells have also recently been demonstrated in synovial membranes, but not in blood of subjects with rheumatoid arthritis, another nonmalignant inflammatory disease (26).

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