γδ T Cell Receptor Analysis Supports a Role for HSP 70 Selection of Lymphocytes in Multiple Sclerosis Lesions

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ABSTRACT

Background: Interactions between γδ T cells and heat shock proteins (HSP) have been proposed as contributing factors in a number of diseases of possible autoimmune etiology but definitive evidence to support this hypothesis has been lacking. In multiple sclerosis (MS), a chronic inflammatory neurologic disease, HSP and γδ T cells are known to colocalize in brain lesions. Analysis of T cell receptor (TCR) gene usage in these lesions has detected evidence of clonality within both the Vδ2-Jδ1 and Vδ2-Jδ3 populations of γδ T cells. In our own studies, using direct sequence analysis, a dominant Vδ2-Jδ3 TCR sequence was found in 9 MS brain samples, suggesting a response to a common antigen. In this report, we have examined γδ T cell receptor gene usage in MS peripheral blood T cell lines selected for reactivity to HSP 70.

Materials and Methods: TCR rearrangement patterns for Vδ2-Jδ1 and Vδ2-Jδ3 were studied using the polymerase chain reaction (PCR) and a direct sequencing technique in populations of peripheral blood mononuclear cells (PBMC) cultured with *Mycobacterium tuberculosis* (*M. tuberculosis*) purified protein derivative (PPD) and then selected for reactivity to a 70-kD heat shock protein (HSP70). Cells were obtained from healthy donors, patients with MS, and patients with tuberculosis (TB). PCR products were subjected to direct sequence analysis to look for evidence of clonality within these T cell lines and to define the sequence of the V-D-J (CDR3) region of the TCR.

Results: In freshly isolated PBMC, both Vδ2-Jδ1 and Vδ2-Jδ3 gene rearrangement patterns were detected, whereas in HSP70^{+} T cell lines the predominant δ chain rearrangement pattern was Vδ2-Jδ3. Direct sequence analyses indicated that in cells reactive with HSP70 the Vδ2-Jδ3 sequences were usually oligoclonal and used Dδ3 exclusively. In four of four MS and two of three TB patients, the oligoclonal sequences in the HSP70^{+} T cell lines were identical to one another and to a dominant sequence previously detected in MS brain lesions. In two of three HSP70^{+} T cell lines from healthy controls, the oligoclonal sequences differed from those found in both groups of patients but were identical to one another except for a small region of heterogeneity in the second N region. In contrast, in freshly isolated PBMC or in PPD[+]HSP70^{−} T cell lines, the Vδ2-Jδ3 gene rearrangement patterns were usually polyclonal and dominant sequences were rarely identified.

Conclusions: These results support the conclusion that a subpopulation of γδ T cells in MS lesions are responding to HSP 70 and that non-CNS-specific antigens contribute to the pathogenesis of MS.

INTRODUCTION

Multiple sclerosis (MS) is a chronic debilitating disease of young adults in which the central nervous system (CNS) becomes the site of an immune attack upon the insulating membrane of nerve fibers, myelin, which is selectively destroyed. While the focus of attention in MS has traditionally been on T cell responses to components of the myelin sheath, evidence is emerging to suggest that non-CNS-specific antigens may play a contributory role, particularly at later stages of the disease. Among these antigens are

the heat shock proteins (HSP), some members of which are known to be strongly immunogenic, conserved across the evolutionary tree, and expressed following exposure to a wide variety of stressors including heat shock and inflammatory mediators (1). Among T cell subsets thought to recognize these antigens are lymphocytes bearing the $\gamma\delta$ T cell receptor (TCR).

$\gamma\delta$ T cells are a minor population of peripheral blood lymphocytes whose exact function remains unclear. In this group of T cells, the repertoire of the V, D, and J region genes has been shown to be limited and junctional diversity is achieved by template and non-template-derived nucleotide insertions in the CDR3 region (2,3). In some tissues, especially in the mouse, $\gamma\delta$ T cells have been shown to express invariant TCR gene usage, suggesting a response to highly conserved antigenic sequences (3). At birth, most $\gamma\delta$ T cells in the peripheral blood and thymus express Vδ1; however, postnatally the Vδ2 cells expand, and in the adult Vδ2/Vγ2 (Vγ9) gene segments are expressed by >70% of circulating $\gamma\delta$ T cells (4,5). These Vδ2 cells also express high levels of CD45RO, a marker for memory T cells, suggesting expansion following exposure to foreign antigens or superantigens (5). Consistent with this hypothesis are the observations that Vδ2-Vγ2-expressing T cells are selectively expanded following exposure to mycobacterial antigens (6–8). Further studies of the $\gamma\delta$ T cell response to mycobacteria have suggested that members of the HSP 65- and 70-kD families may represent immunodominant antigens, although selective expansion of Vδ2 cells in response to mycobacteria-associated superantigens has also been demonstrated (9–13). Mycobacterial HSP 65 and HSP 70 show significant sequence homology with the mammalian HSP 60 and HSP 70 families and the expression of HSP by stressed autologous host cells has implicated a role for immune responses against HSP in diseases of possible autoimmune etiology (9,14). In MS, several studies have suggested the presence of amplified reactivity to recombinant HSP and/or mycobacterial antigens (15–19). The clearest data exist for responses to HSP70, where the relative frequency of HSP70-reactive T cell lines (17) or $\gamma\delta$ T cell clones (18) was found to be significantly increased in peripheral lymphocytes from MS patients compared with healthy donors, patients with TB, and patients with other neurological diseases. $\gamma\delta$ T cells have been localized in demyelinated MS lesions, coincident with in-

creased immunoreactivity for several HSP, suggesting possible functional associations (20,21).

In an attempt to detect the presence of an antigen-driven response within $\gamma\delta$ T cells in MS tissues, several groups have examined TCR gene usage by sequencing and cloning polymerase chain reaction (PCR) products. The results of these studies have shown that, while in the acute stage of the disease Vδ1$^+$ cells accumulate in the CSF and perivascular cuffs, in more chronic stages Vδ2$^+$ cells predominate in demyelinated lesions (20–23). Cloning and sequencing of the PCR products have suggested the presence of clonal expansion in both the Vδ2-Jδ1 and Vδ2-Jδ3 populations of these cells in the lesions, and in Vδ1$^+$ cells in the cerebrospinal fluid (21–24). In our own studies, using direct sequence analysis to assess clonality within MS lesions, the Vδ2-Jδ1 response was found to be polyclonal in eight of nine MS patients, and oligoclonal in one. In contrast, a common dominant Vδ2-Jδ3 TCR sequence was found in all nine MS brain samples, whereas the response for this gene rearrangement pattern was polyclonal in three other neurological disease patients studied for control purposes (24). These data support the conclusion that at least some $\gamma\delta$ T cells in MS lesions respond to a common antigen.

In this report, we have used the same direct sequencing technique to assess TCR gene usage in HSP 70 responsive T cell lines derived from MS patients, tuberculosis (TB) patients, and healthy controls, and have compared the results with those obtained in T cell lines responding to PPD but nonreactive to HSP 70. Since the $\gamma\delta$ T cell responses in MS patients showed predominant usage of Vδ2-Jδ1 or Vδ2-Jδ3 (24), and since evidence of clonality within this population of cells can be most clearly defined by analysis of the δ chain gene (2), we focussed our studies on identification of these TCR rearrangement patterns.

MATERIALS AND METHODS

Donors

All tissue samples and in vitro culture procedures were conducted at the 1st Clinica Neurologica, Universita La Sapienza, Rome, Italy, according to protocols approved by the human experimentation committee of that university. Blood samples were drawn from healthy subjects, patients with relapsing-remitting MS, and patients with bacteriologically or histopathologically proven, active

tuberculosis. Details of the MS patients have been published previously and none had received immunosuppressive therapy for at least 3 months before entering the study (16). The treatment regimen of the TB patients included isonicotinic acid hydrazide (5 mg/kg/day), rifampin (10 mg/kg/day), ethambutol (25 mg/kg/day), and streptomycin (1 g/day for 60 days). None had associated immune deficiencies.

Antigens

Purified protein derivative (PPD) was obtained from the Statens Serum Institut, Copenhagen, Denmark. The recombinant *M. tuberculosis* HSP 70 was obtained from Dr. Jan van Embden (Bilthoven, The Netherlands) through the UNDP/World Bank/WHO program.

Generation of Lymphocyte Lines

Antigen-specific T cell lines were isolated and expanded as described (16). Briefly, peripheral blood mononuclear cells (PBMC) were seeded in 100 μl volumes at a density of 2×10^6 cells/ml in medium (RPMI plus 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% heat inactivated pooled AB serum) in the presence of PPD (10 μg/ml) and expanded with 30 units/ml recombinant human IL-2 every 3–4 days for 15 days. Cultures were restimulated with antigen and 1.5×10^5 irradiated (400 grays) autologous PBMC/well every 15 days.

Proliferation Assays

T cell lines were screened for their proliferative responses to *M. tuberculosis* HSP 70 at the time of the third restimulation with antigen (approximately 45 days in culture). In all proliferation assays, samples of 2×10^4 cells were placed in culture in duplicate or triplicate with 1.5×10^5 irradiated autologous PBMC/well and HSP 70. After 72 hr in culture, the cells were labeled with [^{3}H]-thymidine (0.5 μCi/well) and harvested 16 hr later. A stimulation index of >2 with a Δ cpm >500 was regarded as a positive response.

RNA and cDNA Isolation

Total RNA was extracted from at least 2×10^6 cells using the Chomczynski guanidinium-thiocyanate method. First strand cDNA was synthesized from 5 μg of total RNA in the presence of oligo-dT primers and AMV-reverse transcriptase

(Perkin Elmer Cetus, Emeryville, CA, U.S.A.). PCR amplification was performed using 5 U Taq polymerase in 100 μl reaction mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 2% of the cDNA obtained from 5 μg of total RNA, and 1.0 mM of dATP, dCTP, dGTP, and dTTP. The cDNA amplification was performed for 30 cycles. Each cycle included denaturation at 94°C for 1 min, annealing at 55°C for 2 min and elongation at 72°C for 2 min with an extra cycle of 10 min at 72°C. Following amplification, the samples were separated on a 1% agarose gel. Twenty picomolar of each of the following primers were used: Vδ2 5' ACCCT-CAGGTGCTCCATGAA 3'; Jδ1 3' TCCACAGTCA-CACGGGTTC 5'; and Jδ3 3' TCCACGAAGAGTT-TGATGCC 5'. To rule out the possibility that the nucleotide sequence that we obtained was affected by the primers used, we performed similar experiments with the following internal primers: Vδ2 5' GCACCATCAGAGAGAGATAAGGG 3' and Jδ3 3' AAAACATCTGTCGGGTGTCCC 5'. Primers for β-actin were used as a positive control. The restriction site for Vδ2 was SAL-I and for Jδ1 and Jδ3 was ECO-R1. Details of primer preparation and use, including verification by sequencing and cloning the PCR products, have been reported previously (11).

Direct Sequencing

Direct sequencing was performed from the PCR product according to previously described protocols (24). DNA was purified by isopropanol precipitation to remove excess primer and the DNA was sequenced using a modified Sanger method based on asymmetrical amplification using a primer labeled with [γ^{32}P] ATP with high specific radioactivity (5000 Ci/mmol, 10 mCi/ml) and sequencing reagents from GIBCO-BRL (Gaithersburg, MD, U.S.A.).

RESULTS AND DISCUSSION

The results of the PCR analysis and direct sequencing are shown in Fig. 1 and Table 1. They indicate that, in freshly isolated PBMC, both Vδ2-Jδ1 and Vδ2-Jδ3 gene rearrangement patterns were detected. In contrast, in the PPD$^+$ HSP 70$^+$ T cell lines, the Vδ2-Jδ3 gene rearrangement pattern predominated with only one T cell line expressing both Vδ2-Jδ3 and Vδ2-Jδ1. Examples of the PCR analysis for Vδ2-Jδ1, Vδ2-Jδ3, and β-actin in the PPD$^+$ HSP 70$^+$ T cell lines estab-

FIG. 1. PCR analysis of Vδ2-Jδ1 and Vδ2-Jδ3 in freshly isolated PBMC, or in cells cultured with PPD and selected for reactivity to HSP70

In Panel A, lanes from the left contain molecular weight markers followed by PCR analysis for Vδ2-Jδ1, Vδ2-Jδ3, and β-actin for one MS patient, one healthy control, and one TB patient, respectively, for PPD$^+$HSP70$^+$ T cell lines. In Panel B, lanes from the left contain molecular weight markers followed by PCR analysis for one healthy control for Vδ2-Jδ1, Vδ2-Jδ3, and β-actin for freshly isolated PBMC cultured alone, PPD$^+$HSP70$^+$ and PPD$^+$HSP70$^-$ T cell lines.

lished from one MS patient, one healthy control, and one TB patient are shown in Fig. 1A. The PCR analyses for Vδ2-Jδ1, Vδ2-Jδ3, and β-actin for freshly isolated PBMC, and PPD$^+$ T cell lines reactive or nonreactive for HSP 70 from one healthy control are shown in Fig. 1B.

To determine whether clonality occurred within the Vδ2-Jδ3 gene rearrangement patterns in these T cell lines, the PCR products were subjected to direct sequence analysis. The advantage of this sequencing strategy for TCR analysis is that conserved sequences can be observed as dominant nucleotides, distinguishable from the nucleotide diversity associated with multiple transcripts. Examples of direct sequence analyses for the Vδ2-Jδ3 PCR products are shown in Fig. 2. Analyses for the freshly isolated PBMC, and the HSP 70$^+$ and HSP 70$^-$ T cell lines for one healthy control are shown in the upper panel of Fig. 2, and for freshly isolated PBMC and a HSP 70$^+$ T cell line from one TB patient, and a HSP 70$^+$ and HSP 70$^-$ T cell line from one MS patient are shown in the bottom panel of Fig. 2. As can be seen in this figure, direct sequences derived from the HSP 70$^+$ T cell lines were remarkable for the clarity with which the sequences could be read throughout the junctional region, suggesting oligoclonality within this TCR gene population. In contrast, the direct sequences of the HSP 70$^-$ T cell lines, like the freshly isolated PBMC,

TABLE 1. PCR and direct sequence analyses of cell lines^a

Sample	PBMC		PPD$^+$HSP70$^+$		PPD$^+$HSP70$^-$	
	Vδ2-Jδ3	Vδ2-Jδ1	Vδ2-Jδ3	Vδ2-Jδ1	Vδ2-Jδ3	Vδ2-Jδ1
MS #1	+/P	+	+/O*	−	−	−
MS #2	−	+	+/O*	+	+/P	−
MS #3	+/P	+	+/O*	−	−	+
MS #4	+/P	nd	+/O*	−	na	na
TB #1	na	na	+/O	−	+/O	+
TB #2	+/P	+	+/O*	−	+/P	−
TB #3	+/P	na	+/O*	−	+/P	−
CON #1	+/P	+	+/O**	−	+	+
CON #2	+/P	+	+/O**	−	+/P	+
CON #3	−	+	−	−	−	−

^{a}Presence or absence of bands following PCR analyses for the defined V-J genes are marked by a + and − respectively. Direct sequence analyses were defined as polyclonal (P) or oligoclonal (O), with asterisks marking similarities in the oligoclonal sequences. na, not available; nd, not done.
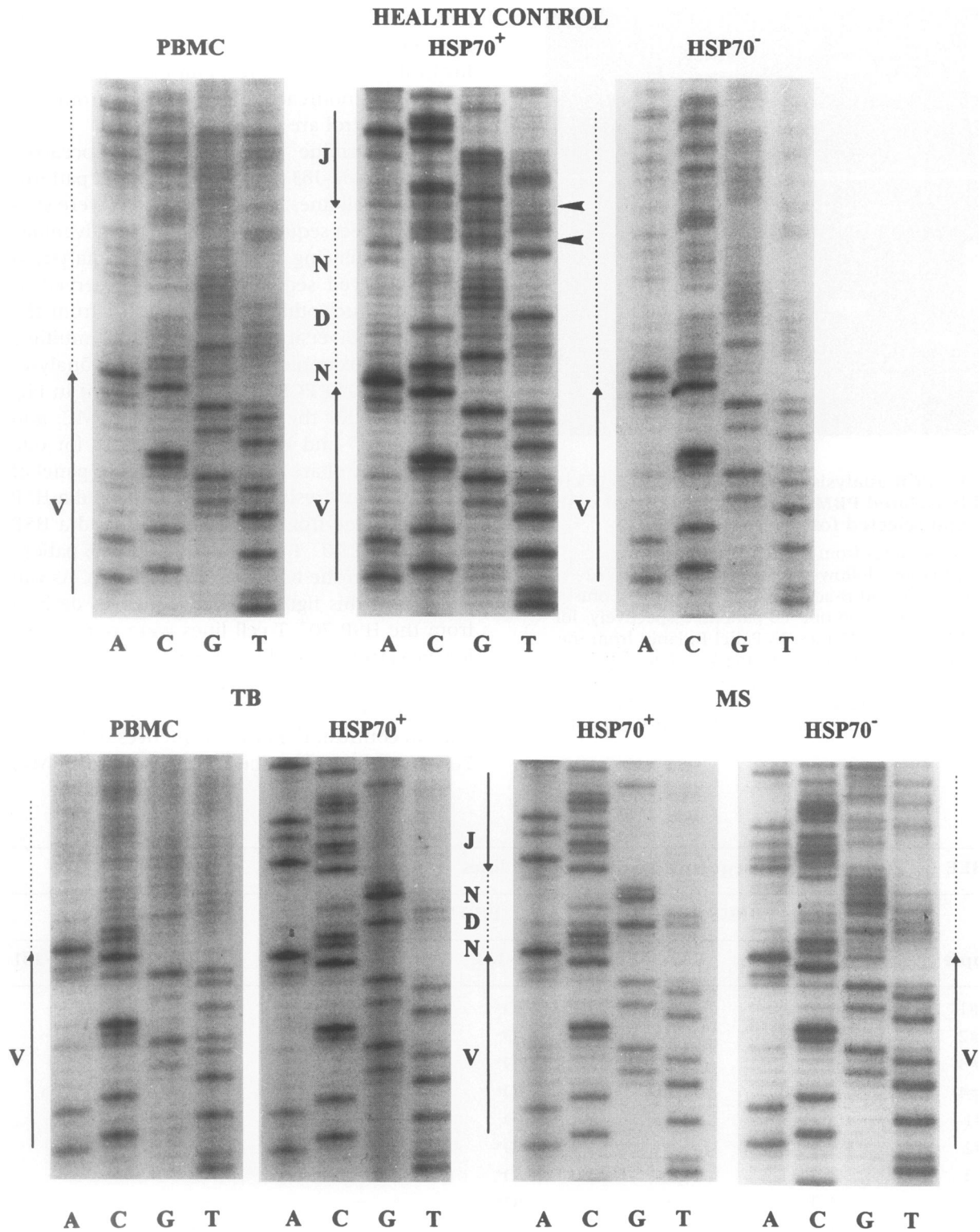
FIG. 2.   Direct sequence analysis of PCR products for Vδ2-Jδ3

(Upper panel) Direct sequence analyses for Vδ2-Jδ3 PCR products from one healthy control for freshly isolated PBMC, PPD⁺HSP70⁺ and PPD⁺HSP70⁻ T cell lines are shown. (Lower panel) Direct sequence analyses for Vδ2-Jδ3 PCR products from one TB patient for freshly isolated PBMC and PPD⁺HSP70⁺ T cell lines, and from one MS patient for PPD⁺HSP70⁺ and PPD⁺HSP70⁻ T cell lines are shown.

TABLE 2. Nucleotide and deduced amino acid sequences detected by direct sequencing

Sample	Vδ2	P/N	Dδ	N	Jδ3
germ-line	TGT GAC ACC				C TCC TGG GAC AC
			δ1 GAAATAGT		
			δ2 CCTTCCTAC		
			δ3 ACTGGGGGATACG		
PPD⁺HSP70⁺					
CONT #1#2	TGT GAC ACC	GTG	CTG GGG GAT ACG	NNN G	CC TGG GAC AC
	C D T	V	L G D T	(*)	A W D T
PPD⁺HSP70⁺					
MS #1,2,3,4	TGT GAC ACC	GTC	GGA	CAC C	AC AC
TB #2, #3	C D T	V	G	H	H T
PPD⁺HSP70⁺	TGT GAC A	GC CGT	TGG G	CA CAC GCA	TCC TGG GAC AC
TB #1	C D	S R	W	A H A	S W D T
PPD⁺HSP70⁻	TGT GAC ACC	G	CT GGG GGG	CG	C TCC TGG GAC AC
TB #1	C D T		A G G	R	S W D T

Vδ2-Jδ3 junctional sequences from direct sequence gels of PCR products from freshly isolated PBMC or cells cultured with PPD and tested for reactivity to HSP70. Sequences obtained from HSP70⁺ T cell lines from controls 1 and 2 differed from one another by only a short region of heterogeneity. Sequences obtained from HSP70⁺ T cell lines from MS patients 2, 3 and 4, and TB patients 2 and 3 were identical. Sequences obtained from an HSP70⁺ and an HSP70⁻ T cell line from one TB patient were oligoclonal but differed from all other sequences.

became unreadable at the end of the Vδ2 sequence and the beginning of the junctional region, indicating the heterogeneity of the transcripts to be expected in the putative CDR3 region of a polyclonal T cell population. The dominant sequences identified in the Vδ2-Jδ3 transcripts, along with the deduced amino acid sequences, are shown in Table 2. All dominant sequences were in frame, displayed portions of the Dδ3 gene segment, and predominantly used the palindromic G at the beginning of the junctional region.

The dominant sequences fell into three groups. In controls 1 and 2, the oligoclonal sequences in the HSP 70⁺ T cell lines were different from those detected in the patients, but were identical to one another except for a small region of microheterogeneity present in the second N region between the Dδ3 and Jδ3 segments (arrows in Fig. 2, top panel). In four of four MS and two of three TB patients, the dominant Vδ2-Jδ3 sequences detected in the HSP 70⁺ T cell lines were identical to one another and to a dominant sequence previously detected in MS brain tissues (24). In the remaining TB patient, the oligoclonal patterns for both the HSP 70⁺ and HSP 70⁻ T cell

lines were different from all of the others. In the two controls, the LGDT sequence in the junctional region represents the second reading frame of Dδ3, whereas in the patients there was a shift to an abbreviated use of the third reading frame of Dδ3. Most dominant sequences also expressed the strongly hydrophobic valine at position 97 of the δ chain (25). Frequent usage of this amino acid (or less often Leu or Ile) at this position in T cells using the Vδ2 gene has been extensively documented (11,23,26,27), particularly in cells expressing Vδ2-Vγ2 gene segments. The expression of this amino acid at this position has been shown to be coded by template-independent N nucleotides and not to be due to pairing constraints (26), suggesting an antigen-driven selection process.

The preferential use of the downstream Dδ3 element in subpopulations of γδ T cells has also been noted previously. In fetal thymic and liver development, the Dδ3 gene is used almost exclusively (5,28), and repeated use of Dδ3 has been noted in Vδ2⁺ cells in human leishmaniasis lesions (29) and in cells cultured from jejunic and colonic biopsies from healthy individuals (30). Interestingly, in the latter study, frequent rear-

rangement to Jδ3 was also noted, as has been found in fetal liver γδ T cells (28). In the adult, although Vδ2 is found on the majority of peripheral blood γδ T cells, rearrangement to Jδ3 is relatively rare and Jδ1 is preferentially used. However, it has been noted that peripheral blood Vδ2 junctions often display greater usage of both Dδ3 and Jδ3, and fewer N nucleotide incorporations than Vδ1 expressing T cells (2,3).

The above results support the conclusion that HSP 70 selects for an oligoclonal response within the Vδ2-Jδ3 population, but whether this represents direct responsiveness to HSP 70 cannot be determined from the present culture conditions. Although some studies have documented a proliferative response of γδ T cells to HSP (9,18), others have suggested that these cells expand in response to activation markers or growth factors associated with T or B cell responses, as for example in the polyclonal activation of Vδ1$^+$ cells to EBV-transformed or activated B cells (31). However, the results of the direct sequencing technique provide compelling evidence for a specific selection process in cells responding to HSP 70. Whereas the sequences in the freshly isolated PBMC or PPD$^+$ HSP 70$^-$ Vδ2-Jδ3 population became unreadable at the end of Vδ2, indicating the variability to be expected in the junctional region, HSP 70$^+$ T cell lines expressed a dominant sequence that was clearly readable throughout the V-D-J region. Oligoclonal expansion of γδ T cells has been noted in a number of pathologic conditions including skin lesions in patients with sarcoidosis (32), leprosy (33), and American leishmaniasis (29); in the joints of patients with rheumatoid arthritis (34), in MS lesions (21,22,24), and in one case of polymyositis in which, like our present findings, there was an oligoclonal response in a Vδ2-Jδ3 population (35). Interestingly, frequent exclusive recombination of Vδ2 and Dδ3 was also noted in some of these tissues (24,34,35). Although evidence of clonality within the peripheral blood population was not noted in many of these studies, a tendency towards oligoclonality within both the Vδ2 and Vδ1 populations has been noted in association with normal aging (36).

A particularly surprising outcome of our studies was the observation that the sequence in four of four MS patients and two of three TB patients was identical to that found in this same subset of γδ T cells in MS lesions. These data suggest that in certain chronic inflammatory diseases, an epitope of HSP 70 selects for a specific subpopulation of γδ T cells, and supports the conclusion that in MS lesions these cells are responding to HSP 70. Although we can only speculate on the potential relevance of this observation to lesion development, γδ T cells are known to possess potent cytotoxic activity and have been demonstrated to kill oligodendrocytes in vitro (37). Alternatively, interaction of γδ T cells with HSP 70 could function in an immunoregulatory pathway following expression of HSP 70 on the surface of inflammatory cells (38). In active MS lesions, immunoreactivity for HSP has been found on inflammatory cells in the perivascular cuffs and on glial cells at the edge of the lesion (20,21), and cytokines have been shown to upregulate HSP 70 on glial cells in vitro (39). Furthermore, a particular polymorphism of HSP 70 has recently been associated with cellular reactivity to HSP 70 in patients with MS (40). HSP 70 is known to be an immunodominant antigen that contains many stimulatory epitopes. Ongoing studies will determine whether the different oligoclonal sequences detected in the patient and control sample groups represent responses to different epitopes of HSP 70 and whether these reside within conserved or nonconserved regions of the protein.

ACKNOWLEDGMENTS

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