Selection of CTL Escape Mutants in Mice Infected with a Neurotropic Coronavirus: Quantitative Estimate of TCR Diversity in the Infected Central Nervous System[1]

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Variant viruses mutated in the immunodominant cytotoxic T cell epitope surface (S) glycoprotein S-510-518 are selected in mice chronically infected with mouse hepatitis virus, strain JHM. We determined whether this selection occurred in the presence of an oligoclonal or polyclonal T cell response using soluble MHC/peptide tetramers in direct ex vivo analyses of CNS-derived lymphocytes. A total of 42% (range, 29–60%) of CD8 T cells in the CNS of mice with acute encephalitis recognized epitope S-510-518. A total of 34% (range, 18–62%) of cells from mice with hind limb paralysis (and chronic demyelination) were also epitope specific, even though only virus expressing mutated epitope is detected in these animals. Sequence analysis of the β-chain CDR3 of 487 tetramer S-510-518-positive cDNA clones from nine mice showed that a majority of clonotypes were identified in more than one mouse. From these analyses, we estimated that 300–500 different CD8 T cell clonotypes responsive to epitope S-510-518 were present in each acutely infected brain, while 100–900 were present in the CNS of each mouse with chronic disease. In conclusion, a polyclonal CD8 T cell response to an epitope does not preclude the selection of T cell escape mutants, and epitope-specific T cells are still present at high levels even after RNA-encoding wild-type sequence is no longer detectable. *The Journal of Immunology,* **1999, 163: 6106–6113.**

The CD8 T cell response is critical for clearance of most viruses, including the neurotropic JHM strain of mouse hepatitis virus (MHV-JHM)[3] (1–3). MHV-JHM causes acute encephalitis and acute and chronic demyelinating diseases in susceptible strains of mice and rats (2, 4). Mice infected with this virus develop a disease with clinical and histological similarities to the human disease, multiple sclerosis. Two CD8 T cell epitopes, encompassing residues 510–518 and 598–605 of the surface (S) glycoprotein (epitope S-510-518 [CSLWNGPHL] and epitope S-598-605 [RCQIFANI], respectively) are recognized in infected C57BL/6 mice. Epitope S-510-518 is the immunodominant of the two epitopes (5, 6).

Infectious virus can be isolated from MHV-infected mice with chronic demyelination under some experimental conditions (7–9). Using one such model, we showed previously that the RNA sequence-encoding epitope S-510-518 was mutated in nearly all samples of both infectious virus and total viral RNA harvested from brains and spinal cords of infected mice with chronic demyelination (10). However, no mutations were detected in epitope S-598-605 nor in the regions flanking the T cell epitopes. Mutations in epitope S-510-518 were evident by 10–12 days postinoculation (p.i.), and their presence resulted in significant loss of recognition by CNS-derived CTL in direct ex vivo cytotoxicity assays (10, 11). In addition, infection of naive mice with these CTL escape mutants resulted in decreased virus clearance and increased mortality and morbidity (12).

CTL escape mutants have been detected in several infections of humans and experimental animals, but their selection is not a common phenomenon (1, 13, 14). In the situations in which CTL escape mutants appear to be important (15, 16), the immune response is strongly focused on a single epitope. It has been suggested that CTL escape mutants are selected only in the presence of a monospecific CD8 T cell response characterized by limited TCR diversity (13).

Direct ex vivo analysis of the TCR diversity of Ag-specific T cells has been facilitated by the recent development of MHC/peptide tetramers able to differentiate CD8 T cells with differing specificities (17). Using these reagents, it is possible to determine directly clonality within the infected CNS. The clonality of the response is often assessed by analysis of the complementarity-determining region 3 (CDR3) of the TCR. The TCR is a heterodimer consisting of an α- and β-chain. The great diversity in the T cell response results from the large number of different V, D, and J elements in the germline coupled with imprecise joining at the V-D and D-J junctions (β-chain) or V-J junction (α-chain) (18). These junctional regions are encompassed by the CDR3 of the α- and β-chains and make direct contact with the MHC/peptide complex (19). Both the length and sequence of this region are important in Ag specificity.

In recent analyses of several infections, it has been shown that TCR repertoire diversity is similar during the primary response and in the memory pool (20–22). This response is usually polyclonal, although there is often striking preferential usage of

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[3] Abbreviations used in this paper: MHV, mouse hepatitis virus; MHV-JHM, mouse hepatitis virus, strain JHM; CDR3, complementarity-determining region 3; S, surface glycoprotein; p.i. postinoculation.

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specific Vβ and Jβ elements and conservation of length of the CDR3. Furthermore, even relatively small phenotypic and functional changes during secondary responses may result in increased specificity and avidity (22–24).

CD8 T cells are expanded within a few days of infection with MHV-JHM and are continuously exposed to Ag until clinical disease develops. CTL escape mutants are isolated from MHV-JHM-infected mice several weeks after initial exposure to virus, with wild-type sequence encoding this epitope only rarely detected in mice with chronic demyelination (10). The influence of increasing viral Ag, albeit with mutations abrogating T cell recognition, provides a unique model to study alterations in the TCR repertoire, as stimulation of epitope S-510-518-specific T cells is expected to diminish with the loss of the wild-type epitope sequence. Therefore, this model of chronic demyelination associated with persisting infectious virus was used to analyze changes in TCR expression during progressive disease.

Materials and Methods

Animals

MHV-seronegative C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and the National Cancer Institute (Bethesda, MD). To obtain animals with acute encephalitis, 6-wk-old mice were inoculated intranasally with 4×10^4 PFU MHV-JHM. The strain of MHV-JHM used in these studies is highly virulent, and infection of naive C57BL/6 mice with this virus results in a uniformly fatal acute encephalomyelitis. Mice were sacrificed when moribund (6–7 days p.i.). To obtain mice with chronic demyelination, suckling mice were inoculated intranasally with 4×10^4 PFU MHV-JHM and nursed by dams previously immunized with live MHV-JHM as previously described (7). Mice were sacrificed when they developed hind limb paralysis, at times p.i. indicated in the figures and tables.

Viruses

MHV-JHM was grown and titered as previously described (7). A CTL escape mutant (mutated in residue 514 [Asp to Ser] of the S glycoprotein) was isolated and propagated as previously described (12).

Abs

Ab to the Fc receptor (2.4G2), Cy5-conjugated Abs to CD4 (GK1.5), IgM (B76) and Mac-1 (M1/70), and FITC-conjugated anti-CD8 Ab (Lyt-2) were obtained as described previously (25). Biotinylated monoclonal anti-mouse Vβ TCR Abs (Vβ2, clone B20.6; Vβ3, KJ25; Vβ4, KT4; Vβ5.1, 5.2, MR9-4; Vβ6, RR4-7; Vβ7 TR310; Vβ8.1, 8.2, MR5–2; Vβ9, R10-2; V$\beta10^b$, B21.5; Vβ11, RR3-15; Vβ12, MR11-1; Vβ13, MR12-3; Vβ14, 14-2) were all purchased from PharMingen (San Diego, CA).

Isolation of lymphocytes from the MHV-infected CNS

Lymphocytes were isolated from the infected CNS as previously described (26). In brief, mice were perfused with PBS, and the brains and spinal cords were removed. Tissue was ground between frosted glass slides and triturated by vigorous pipetting in 5 ml of RPMI 1640 medium with 10% FCS. Following thorough tissue dispersion, Percoll (Pharmacia, Uppsala, Sweden) was added to a final concentration of 30%. The lysate was centrifuged at $1300 \times g$ for 30 min at 4°C. The Percoll and lipid layers were aspirated and the cell pellet was washed and resuspended in 5 ml RPMI 1640 medium with 10% FCS. The cells were layered over 2 ml Lympholyte-M (Cedarlane Laboratories, Homby, Ontario, Canada) and centrifuged at $1300 \times g$ for 20 min at room temperature. Cells were removed from the interface, washed, and counted.

Tetramers

Biotinylated MHC class I (H-2D^b) monomers complexed with peptide S-510-518 were produced as described previously (27). Biotinylated monomers were tetramerized with avidin-PE (Vector Laboratories, Burlingame, CA).

FACS analysis

The TCR phenotypes of CD8 T lymphocytes from brains infected with MHV-JHM were determined by four-color FACS analysis. Briefly, 100–300,000 cells were first incubated with Ab to the Fc receptor (mAb 2.4G2) in rat serum. This was followed by triple staining with a mixture of Cy5-conjugated Abs to CD4 (GK1.5), IgM (B76) and Mac-1 (M1/70), FITC-conjugated anti-CD8 Ab (Lyt-2), and PE-conjugated S-510-518-specific MHC class I tetramers (tetramer S-510). After extensive washing, cells were incubated with biotinylated monoclonal anti-mouse Vβ TCR Abs and were then incubated with Texas red-conjugated streptavidin. Cells were analyzed with an EPICS 753 (Beckman Coulter, Fullerton, CA).

For analysis of sorted cells, cells from the CNS of a single animal were stained as above with the Cy5-conjugated Ab mixture, FITC-conjugated anti-CD8 Ab, and PE-conjugated tetramer S-510. Tetramer S-510-positive CD8 T cells were sorted on an EPICS 753. A total of 4,000–20,000 tetramer S-510-positive CD8 T cells were obtained after sorting.

Isolation of RNA from lymphocytes.

RNA was isolated from lymphocytes using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the specifications of the manufacturer.

Sequence analysis

In all cases, cDNA was synthesized from 2 μg lymphocyte RNA as previously described (10). For the synthesis of Vβ13-specific cDNA clones, a Vβ13-specific primer (CCTAAAGGAACTAACTCCACTCT) was used in conjunction with a common Cβ reverse primer (GCAATCTCTGCTTTTGATGGCTC) to prepare PCR products. The primers contained GCG clamps and restriction sites (Cβ primer, *Eco*RI site; Vβ primer, *Bam*HI site) to facilitate cloning of PCR products into pIBI31 (IBI, New Haven, CT). DNA was prepared from cDNA clones using QIAprep Spin Miniprep Kits (Qiagen) or CONCERT Rapid Plasmid Miniprep Systems (Life Technologies, Gaithersburg, MD). Clones were sequenced using an automated sequencer (ABI 373A Stretch Sequencer; Applied Biosystems, Foster City, CA) with T7 (TAATACGACTCACTATAGGG) and T3 (CTGTAATTAACCCTCACTAAAG) promoter primers.

Estimate of repertoire size

A logarithmic distribution has the form $s_i = \alpha x^i/i$ (28–31), where s_i is the number of CDR3 expected to be represented i times, α is an index of clonal diversity, and x is a parameter related to the sample size (number of cDNA clones sequenced). This distribution is often used to describe data in which there are a few abundant species and many rare species (29, 31). In our analyses, a logarithmic series is characterized by two parameters: S, the total number of different CDR3 species present in the Vβ13 tetramer S-510-positive CD8 T cell population, and N, the total number of Vβ13 tetramer S-510-positive CD8 T cells in the infected CNS. These two are related by a constant, α, which is a measure of diversity of CDR3 usage: $S = \alpha \ln(1 + N/\alpha)$. The value of α is high if the number of different CDR3 is high relative to the number of individuals and low if the number of different CDR3 is low. Logarithmic distributions were fit to our data using a computer program written in QuickBASIC (version 4.5; Microsoft, Redmond, WA). For each mouse, the goodness-of-fit of the estimated logarithmic distribution to the data was assessed using a G test of independence with Williams' correction (32), using observed and expected counts for five frequency classes (sequences represented once, 2–3 times, 4–7 times, 8–15 times, and 16 or more times). SEs for our α estimates were obtained following Krebs (30).

Results

The CD8 T cell response to epitope S-510-518 is polyclonal in the CNS of mice with acute encephalitis

In mice infected i.p. with MHV-JHM, splenocytes expressing a diverse group of Vβ elements recognize epitope S-510-518. Sequence analysis of β-chain CDR3 revealed a polyclonal response (25). These results suggested that CTL escape mutants were selected in the presence of a polyclonal CD8 T cell response. However, as the CNS is the site of infection in mice with disease caused by MHV-JHM, CTL escape mutants likely arise from immune pressure in the CNS and not periphery. Therefore, the polyclonality of CD8 T cells in the CNS was investigated to confirm the broad TCR spectrum observed in peripheral organs.

Soluble MHC class I/peptide S-510-518 tetramers were constructed as previously described (17) and used to stain lymphocytes isolated from the CNS of mice with acute encephalitis. Negative controls included CD8 T cell lines not responsive to epitope S-510-518 and splenocytes from uninfected mice. As a positive

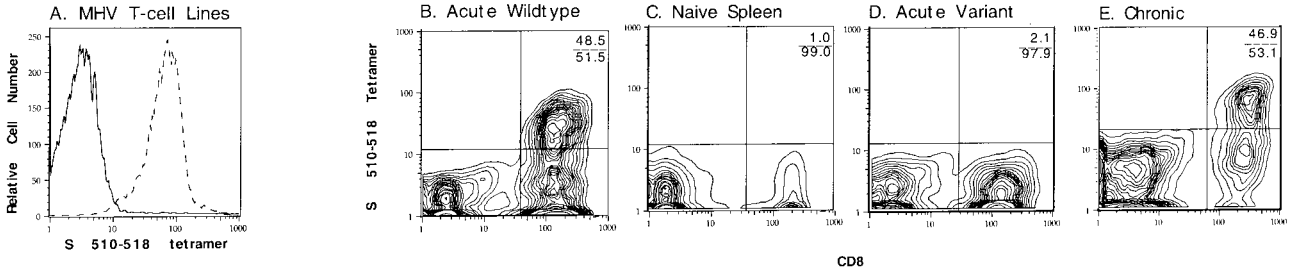

FIGURE 1. Specificity of staining with tetramer S-510. Samples were stained for surface CD8 and with tetramer S-510 as described in *Materials and Methods*. *A*, Epitope S-510-518-specific (dashed line) and epitope S-598-605-specific (solid line) CD8 T cell lines were stained with tetramer S-510. *B–E*, Staining of lymphocytes harvested from the CNS of a mouse with MHV-JHM-induced acute encephalitis (*B*), a naive spleen (*C*), the CNS of a mouse with acute encephalitis induced by virus mutated in epitope S-510-518 (*D*), and the CNS of a mouse with chronic demyelination harvested at 25 days p.i. (*E*). The numbers given show the percentage of CD8 T cells that are tetramer S-510-positive and tetramer S-510-negative. The experiments shown in *B*, *D*, and *E* were performed on different days. Only the negative control for *B* is shown (*C*).

control, a CD8 T cell line recognizing epitope S-510-518 was analyzed. As shown in Fig. 1*A*, tetramer S-510 bound specifically to the epitope S-510-518-specific CD8 T cell line but did not stain cells harvested from a CD8 T cell line recognizing epitope S-598-605. Tetramer S-510 bound to 42% (range, 29–60%; $n = 7$ groups of three to six mice each) of the CD8 T cell population from mice with acute encephalitis. Staining was specific because the reagent did not stain naive splenocytes (Fig. 1*C*) or CD8 T cells harvested from the CNS of a mouse infected with a variant expressing a mutation in epitope S-510-518 (CSLW*S*GPHL) (Fig. 1*D*). The fraction of CD8 T cells recognizing epitope S-510-518 in the infected CNS is remarkably elevated, but consistent with results obtained from analyses of several human and experimental infections (27, 33, 34).

To determine whether the CNS-derived CD8 T cells specific for epitope S-510-518 were polyclonal, Vβ-specific Abs were used to analyze the TCR Vβ repertoire. For these experiments, CNS-derived lymphocytes were harvested from pools of three to six mice, because insufficient numbers of cells were obtained from single animals for a complete set of analyses. Preliminary experiments showed that some Vβ-chains were used relatively infrequently, so pools of anti-Vβ Abs were used for staining to conserve cells. Staining for representative TCR Vβ expression is shown in Fig. 2, and the results are summarized in Table I. TCR Vβ usage by tetramer S-510-positive T cells was very diverse with a distribution that differed from that found in the tetramer S-510-negative population and in the naive spleen. Epitope S-510-518-specific CD8 T cells expressing the Vβ13 element were over represented when compared with TCR usage by naive spleen cells although both tetramer S-510-positive and tetramer S-510-negative cells preferentially expressed this element (Fig. 2). As also shown in Fig. 2, cells expressing the Vβ8 element were preferentially expressed in the tetramer S-510-positive population whereas Vβ5 expression was over represented in the tetramer S-510-negative population. These results are similar to what was observed in previous analyses of epitope S-510-518-specific splenocytes harvested from mice after i.p. infection (25).

Although many TCR Vβ-chains were expressed by epitope S-510-518-specific T cells, this response may still in large part be oligoclonal. Clonality of the T cell response was determined by sequence analysis of the β-chain CDR3 of sorted, tetramer S-510-positive CD8 T cells isolated from single mice with acute encephalitis. Vβ13-expressing cells were chosen for analysis because

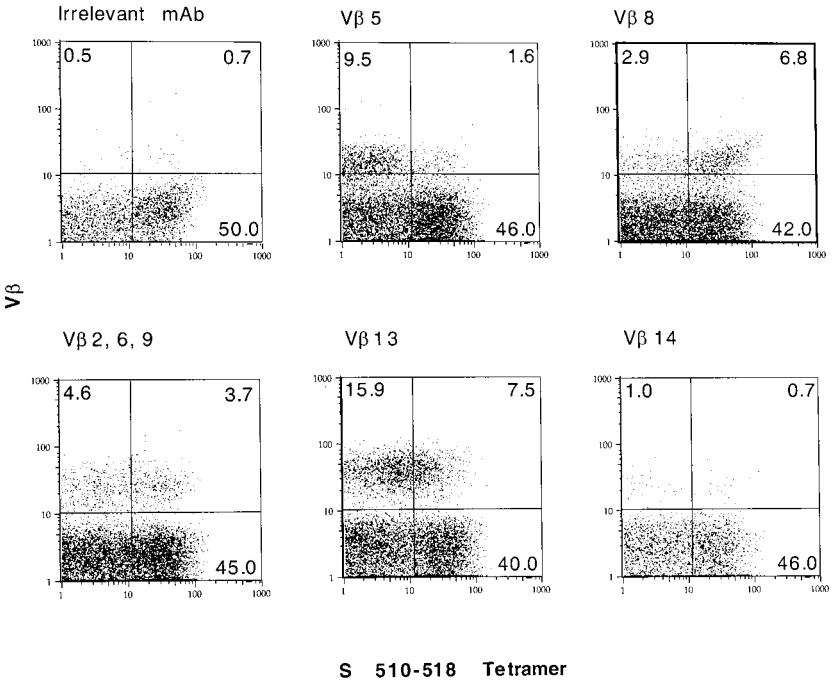

FIGURE 2. TCR usage by epitope S-510-518-specific cells harvested from mice with acute encephalitis. Cells were prepared from three to six mice with acute encephalitis and analyzed for CD8, tetramer S-510, and Vβ expression by FACS as described in *Materials and Methods.* Cells were gated on CD8. Shown are data from a representative experiment. Tetramer S-510-positive cells preferentially express Vβ8, whereas the Vβ5 element is over-represented in the tetramer S-510-negative population. Vβ13 and the pool of Vβ2, Vβ6, and Vβ9 elements were represented in both the tetramer S-510-negative and tetramer S-510-positive populations. The data from five experiments are summarized in Table I.

Table I. *TCR Vβ expression by CD8 T cells from naive spleens and from the infected CNS*[a]

Vβ	Spleen Cells %	% CNS Cells (SE)	
		Tetramer S-510-positive	Tetramer S-510-negative
Vβ2 + Vβ6 + Vβ9	5.6	6.1 (0.7)	10.0 (1.1)
Vβ3	3.4	3.1 (1.7)	0.9 (0.4)
Vβ4	4.0	7.5 (0.8)	3.9 (0.4)
Vβ5	15.3	7.4 (2.2)	19.1 (2.0)
Vβ7	6.5	9.0 (0.6)	5.3 (1.3)
Vβ8	14.8	14.1 (2.4)	4.1 (0.8)
Vβ10	4.4	4.6 (0.2)	2.8 (0.8)
Vβ11 + Vβ12	4.7	4.9 (1.8)	14.6 (2.2)
Vβ13	4.6	22.8 (3.8)	20.0 (2.4)
Vβ14	3.1	0.3 (0.2)	1.9 (0.1)

[a] CD8 T cells harvested from spleens of two naive mice were analyzed. Five groups of three to six mice with acute encephalitis were analyzed in these experiments.

these cells were most abundant in the epitope S-510-518 population (Table I).

Sequence analysis of the TCR Vβ13-expressing cells from three mice showed a polyclonal response with diverse Jβ usage, although there was preferential expression of the TCR Jβ2 family members. Extensive variation in CDR3 length was also apparent. The results obtained from the analysis of 80 cDNA clones derived from a single mouse with acute encephalitis are shown in Table II. Twenty-two different CDR3 were detected with 12 identified only in single clones. Nine different Jβ elements were encoded in these cDNA clones, and CDR3 length varied from 6 to 10 aa. Six of these CDR3 were also detected in at least one other mouse with acute encephalitis (Table II). The abundance data from all three mice are summarized in Table III. In one mouse (acute 3), a single clonotype comprised 50% of all of the epitope S-510-518-specific clones, but the remainder of the clones were very heterogeneous.

A polyclonal response to epitope S-510-518 is retained in mice with chronic demyelination

In persistent infections caused by lymphocytic choriomeningitis virus, HIV-1, and EBV, the T cell repertoire is characterized by continuous selection, deletion of Ag-specific T cells, and loss of effector function (21, 35–38). Next, we determined whether changes in the diversity of the epitope S-510-518-specific TCR repertoire occurred in mice with chronic demyelination.

In the model used in this study, suckling mice were inoculated intranasally with MHV-JHM, and acute encephalitis was prevented by nursing by dams previously immunized with virus. A variable percentage (30–90%) later developed hind limb paralysis. Histological evidence of inflammatory cell infiltration and demyelination was detected in all mice with hind limb paralysis but not in mice that remained asymptomatic (7). Little or no wild-type epitope S-510-518 was detected by the time mice develop hind limb paralysis, and the vast majority of the virus are CTL escape mutants (10). However, although the total number of CNS-derived CD8 T cells was decreased compared with the acute infection, epitope S-510-518-specific CD8 T cells, detected with tetramer S-510, comprised a significant proportion in the CNS of mice with hind limb paralysis (34% (range, 18–62%; $n = 7$ mice)) (Fig. 1*E*). These cells recognize the epitope in cytotoxicity assays and secrete IFN-γ in response to peptide S-510-518 (data not shown).

Because, in general, only single animals with chronic demyelination were available at a time, it was not possible to obtain pools of CNS-derived lymphocytes for flow cytometric analysis of Vβ usage. Rather, we measured the diversity of TCR Vβ13 tetramer S-510-positive CD8 T cells in six mice with chronic demyelination because TCR Vβ13-expressing cells were over-represented in the population of CD8 T cells responding to epitope S-510-518, as in the acutely infected mice. The data, summarized in Table IV, revealed a diverse TCR response, although there was greater variability in the number of clones responding to the epitope than in mice with acute encephalitis. At present, we cannot correlate this difference in diversity with any difference in clinical outcome. As in the mice with acute encephalitis, multiple Jβ elements were expressed in the tetramer S-510-positive T cell population and CDR3 length was variable.

Diversity of sequences within Vβ13 CD8 T cell population responding to epitope S-510-518

The distribution of spleen-derived Vβ13 CD8 T cell cDNA clones responding to epitope S-510-518 was shown empirically to fit a

Table II. *Sequence analysis of TCR Vβ13 CDR3 from mouse (acute 1) with encephalitis*

Clone No.	V	CDR3	J	Jβ	Frequency
1[a]	SG	AFSGNTL	YFG	1.3	13/80
2	SS	FGTSNERL	FFG	1.4	1/80
3	SS	FWGAAPL	YFA	1.6	2/80
4	SS	LGQGNSPL	YFA	1.6	1/80
5	SS	FAGIAEQ	FFG	2.1	3/80
6[a]	SS	LWGGAYAEQ	FFG	2.1	12/80
7[a]	SS	RGGYAEQ	FFG	2.1	1/80
8	SS	FWGGQAEQ	FFG	2.1	1/80
9	SS	HWGGGYAEQ	FFG	2.1	2/80
10	SS	QPGIVYAEQ	FFG	2.1	1/80
11	SS	RTGTNTGQL	YFG	2.2	1/80
12[a]	SS	LGAIWGAETL	YFG	2.3	17/80
13	SS	FFGTGSAETL	YFG	2.3	1/80
14	SS	FPGGNTL	YFG	2.4	3/80
15[a]	SS	FWGTSQNTL	YFG	2.4	7/80
16	SS	SWGSSQNTL	YFG	2.4	1/80
17	SS	PWGGADTQ	YFG	2.5	1/80
18	SS	PWGGGQDTQ	YFG	2.5	1/80
19	SS	FWGGSSYEQ	YFG	2.6	1/80
20[a]	SS	LWGGAHEQ	YFG	2.6	6/80
21	SS	PQGWEQ	YFG	2.6	3/80
22	SS	RLDWGNYAEQ	YFG	2.6	1/80

[a] Detected in at least one other mouse with acute encephalitis.

Table III. *Diversity and abundance of epitope S-510–518-specific Vβ13 CD8 T cells in acutely infected CNS*

Mouse	No. Analyzed[a]	No. Species[b]	Abundance (no. copies)[c]							Common Sequences[d]	
			1	2	3	4	5–7	8–15	>16	Acute	All
Acute 1	80	22	12	2	2	1	2	2	1	6/22	12/22
Acute 2	35	14	11	0	0	0	1	2	0	5/14	11/14
Acute 3	40	18	15	1	1	0	0	0	1	2/18	8/18

[a] Number of cDNA clones analyzed per mouse.
[b] Number of different cDNA clones identified.
[c] Number of cDNA clones within each abundance class.
[d] Number of Vβ13 cDNA clones detected in at least one other mouse with acute encephalitis ("acute") or acute or chronic disease ("all").

logarithmic distribution (25). A value of α (a measure of CDR3 diversity) was calculated for Vβ13 tetramer S-510-positive CD8 T cells for each acutely or chronically animal, as described in *Materials and Methods* (Table V). The distributions of observed and expected abundance of CNS-derived TCR Vβ13 cDNA clones for representative animals are shown in Fig. 3. The logarithmic distribution in each case underestimated the number of unique sequences and the number of very common sequences in the CNS, although only in the case of acute mice 2 and 3 was the difference statistically significant (Table V). However, analyses based on subsampling our observed distributions suggest that errors arising from lack of fit to the logarithmic distribution will be small compared with those arising from sampling uncertainty (data not shown).

The approximate number of Vβ13 CD8 T cells within the infected CNS detected with tetramer S-510 was calculated from these data, recognizing that the resultant number is a minimal estimate because the fitted distribution underestimates unique sequences. There are ~40,000 Vβ13 tetramer S-510-positive CD8 T cells per acutely infected CNS (1.5×10^6 lymphocytes/infected CNS × 28% CD8 × 42% tetramer S-510-positive × 23% Vβ13) and 6,600 per chronically infected CNS (5.3×10^5 lymphocytes/infected CNS × 18% CD8 × 34% tetramer S-510-positive × 20% Vβ13). Using our estimates of α (and their SEs), we calculated that epitope S-510-518 is recognized by a minimum of 73–102 Vβ13 CD8 T cells expressing different TCR β rearrangements in mice with acute encephalitis and 18–161 Vβ13 CD8 T cells in mice with chronic demyelination (Table V). To simplify comparison between different animals, a column showing the number of different cells expressing each TCR Vβ element per 10,000 CD8 T cells is also included in Table V.

From the data in Table V, the total number of different CD8 T clonotypes responding to epitope S-510-518 can be approximately calculated. This calculation assumes that the same diversity is present within each Vβ subrepertoire. If CD8 T cells expressing 73–102 different Vβ13-positive TCR respond to this epitope and these cells represent 23% of the total CD8 T cell population (Table I), ~300–500 epitope S-510-518-specific cells expressing different β-chains are present per mouse with acute encephalitis. In mice with chronic demyelination, ~100–900 different clonotypes recognize epitope S-510-518. This number is likely to be an underestimate for the reasons stated above and also because it does not include the contribution of the TCR α-chain to diversity.

Discussion

The first objective of this study was to determine whether the selection of CTL escape mutants occurs only in the presence of an oligoclonal T cell response. Conversely, we sought to determine whether virus persistence and the appearance of these mutants change the TCR repertoire. Our results show that a polyclonal response does not prevent the selection of CTL escape mutants. The T cell response to epitope S-510-518 is polyclonal, even though this response is in large part monospecific. Thus, although differences in the fine specificity of the response to this epitope have been demonstrated, the net effect is that several mutations in both MHC and TCR contact residues result in a loss of CTL recognition in the infected CNS (10, 25). In a recent report, it was concluded that a polyclonal CD8 T cell response was, in general, multispecific. Therefore, mutations in a single TCR contact residue would not be likely to result in viral escape because not all T cell clones would be inactivated by a single mutation (39). Our results show that this is not true for mice infected with MHV-JHM and that even if the CD8 T cell response is polyclonal, it may be functionally monospecific.

Our results also show that this polyclonality is maintained in mice with hind limb paralysis, even as the relative abundance of wild-type epitope S-510-518 diminishes. Similar numbers of clonotypes/10,000 epitope S-510-518-specific CD8 T cells were detected in mice with acute encephalitis and in those with chronic

Table IV. *Diversity and abundance of epitope S-510–518-specific Vβ13 CD8 T cells in chronically infected CNS*

Mouse[a]	No. Analyzed[b]	No. Species[c]	Abundance (no. copies)[d]							Common Sequences[e]	
			1	2	3	4	5–7	8–15	>16	Chronic	All
Chronic 1 (30)	86	40	27	5	3	1	1	3	0	14/40	19/40
Chronic 2 (29)	68	18	10	5	1	0	1	0	1	12/18	15/18
Chronic 3 (25)	48	7	3	1	0	0	0	1	2	7/7	7/7
Chronic 4 (31)	45	11	7	1	0	0	0	3	0	8/11	10/11
Chronic 5 (25)	42	14	8	2	1	0	1	2	0	12/14	13/14
Chronic 6 (27)	43	20	10	5	3	0	1	1	0	15/20	15/20

[a] Values in parentheses represent day p.i. harvested.
[b] Number of cDNA clones analyzed per mouse.
[c] Number of different cDNA clones identified.
[d] Number of cDNA clones within each abundance class.
[e] Number of Vβ13 cDNA clones detected in at least one other mouse with chronic demyelination ("chronic") or acute or chronic disease ("all").

Table V. *Estimate of number of TCR Vβ13 epitope S-510–518-specific clonotypes in infected CNS*

Sample	Tetramer S-510/CD8 T cells (%)	Estimate of α (p)[a]	Total No. of Different Clonotypes per Infected CNS (CI)[b]	Frequency (per 10,000 cells)
Acute 1	41.4	10.02 (0.748)	83 (51–113)	69
Acute 2	28.4	8.65 (0.0497)	73 (37–106)	61
Acute 3	37.0	12.59 (0.0499)	102 (58–142)	84
Chronic 1	25.8	29.08 (0.203)	158 (115–197)	170
Chronic 2	29.5	7.99 (0.225)	54 (31–74)	57
Chronic 3	46.9	2.26 (0.473)	18 (7–29)	19
Chronic 4	24.8	4.64 (0.12)	34 (15–51)	36
Chronic 5	18.2	7.35 (0.66)	50 (26–72)	53
Chronic 6	30.6	14.5 (0.77)	89 (54–121)	95

[a] Values of p from a G test for goodness-of-fit to the estimated logarithmic distribution; $p < 0.05$ indicates significant departure of the data from the logarithmic distribution.
[b] Numbers assume fit to the estimated logarithmic distributions. As calculated in the text, we estimate that there are ~40,000 Vβ13 epitope 510–518-specific CD8 T cells in the acutely infected CNS and 6,600 Vβ13 epitope 510–518-specific CD8 T cells in the CNS of mice with chronic demyelination. Also included are number of clones expected per 10,000 cells, to simplify comparisons. CI, 95% confidence interval.

demyelination (Table V). However, the total numbers of epitope-specific CD8 T cell clonotypes were lower in the chronically infected mice, because fewer CD8 T cells were present in the CNS when compared with mice with acute encephalitis (Table V). Substantial variation was detected in the size of the TCR repertoire in the chronically infected mice (Tables IV and V). The explanation for this variability in repertoire diversity is not clear. However, it could reflect, in part, intra-animal variability in the naive repertoire or in the kinetics of maturation of the immune response. Alternatively, because no information is available about the specific mutation in epitope S-510-518 selected in each chronically infected mouse, it is possible that different mutations in the epitope have a variable effect on the TCR repertoire.

Common clonotypes were detected in mice with acute encephalitis and in those with chronic demyelination with only a few CDR3 sequences not detected in more than one mouse in the latter group (Tables III and IV). With rare exceptions, most of the sequences detected in only a single animal were present at low abundance. These numbers underestimate the amount of overlap between individual mice, because the probability of detecting a certain CDR3 in each mouse would increase if more cDNA clones were sequenced. These results suggest that the TCR repertoire is fairly stable and that T cell clonal deletion does not occur during MHV persistence. Comparison of Tables III and IV suggests that there is narrowing of the TCR repertoire because fewer clones present in only one animal were detected in the chronic samples. Narrowing of the TCR repertoire has been reported previously and shown to be associated with an increase in TCR avidity (20, 23, 24).

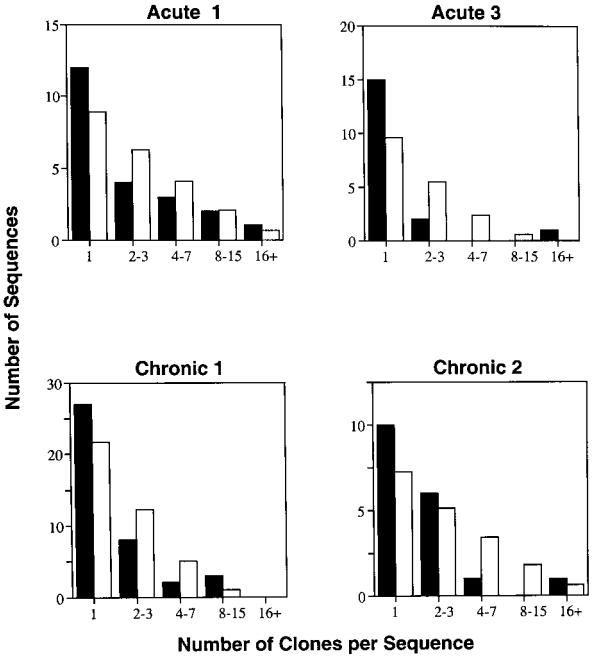

FIGURE 3. Frequency of tetramer S-510-positive Vβ13 T cell cDNA clones in the CNS of mice with acute encephalitis (acute 1, 3) or chronic demyelination (chronic 1, 2). Data obtained from analyses of nine individual mice were used to calculate a value for α for each mouse as shown in Table V. From this value, the predicted abundance of clones was calculated. Frequency distributions for representative samples are shown. Closed symbols, observed values. Open symbols, expected values.

The continued presence of epitope S-510-518-specific CD8 T cells in the absence of wild-type epitope was not predictable. Persistence of CTLs in the apparent absence of any nonmutated target epitope has also been documented in chimpanzees infected with hepatitis C (40). Continued detection of epitope-specific CD8 T cells may reflect stimulation by mutated epitope S-510-518. Although this may seem unlikely because most of the common mutations detected in previous studies (11) abrogate or greatly diminish cytolysis by epitope S-510-518-specific CD8 T cells, engagement of TCRs may still be sufficient to elicit other T cell effector function. These cells would then be present in the CNS at times when wild-type epitope has disappeared. Alternatively, although viral RNA encoding wild-type epitope may not be detected in the chronically infected CNS, viral protein expressing this sequence may still be present and able to stimulate epitope S-510-518-specific CD8 T cells. Prolonged retention of Ag within the CNS has been suggested in other studies (41). It is also possible that virus-encoding wild-type sequence persists in the infected CNS at levels below the sensitivity of our assays but sufficient for stimulation of epitope S-510-518-specific CTLs.

The diversity of the T cell response reflects both the number of different T cell clones and relative abundance of each clone. Because it is not possible to sequence the α and β CDR3 for every Ag-specific CD8 T cell clone in a given responding population, alternative methods to measure the diversity of the TCR response have been developed. In one method that is commonly used, spectratyping, the repertoire of T cell clones expressing each Vβ element (21 different TCR Vβ elements are expressed in the mouse) is analyzed separately (42). Using reverse transcription PCR, Jβ usage and/or CDR3 length profiles are determined for T cell populations expressing each Vβ or Vβ-Jβ subrepertoire. The profile of Vβ and Jβ usage as well as length of the CDR3 are skewed in the CD8 T cell response to many pathogens when compared with similar measurements made on naive populations of CD8 T cells (21,

22). Spectratyping tends to focus on the most abundant components of the T cell response and less so on its diversity.

In this report, a direct sequencing method was used to measure TCR diversity. We sequenced the CDR3 from 35 to 86 tetramer S-510-positive CD8 T cell cDNA clones per mouse. This type of analysis has been used previously (43, 44). It provides information about both the diversity and abundance of the T cell clones responding to this Ag and complements analyses using spectratyping (21, 22, 42). In a preliminary set of analyses using Vβ13 T cell clones from mouse acute 1, we found that the values for α, the index of diversity, did not change significantly as the number of cDNA clones analyzed increased from 40 to 80. Consequently, we sequenced a reduced number of clones in subsequent samples.

Naumov et al. (44), using methodology similar to ours, analyzed 294 Vβ17 TCR CD8 T cell cDNA clones harvested from the peripheral blood of a human responding to an epitope within the matrix (M1) protein of influenza A (M-58-66). Within these 294 clones, 95 different sequences were identified with 61 detected only once. When these data were fitted to a logarithmic series, an α value of 49.62 (α is the index of diversity) was calculated. These data, like the data that we obtained from samples acute 2 and acute 3 (Fig. 3), differed significantly from a logarithmic distribution in having more clones than expected detected only once. With this caveat, we used this value of α to estimate that 264 different sequences would be expected (95% confidence interval, 218–306) per 10,000 Vβ17 CD8 T cell clones present in this population. This is higher than the equivalent number calculated for the Vβ13 CD8 T cell population in the MHV-infected CNS. However, most of the CD8 T cells responding to epitope M-58-66 express the Vβ17 element (45), whereas only a minority of the MHV-specific CD8 T cells express any single TCR Vβ gene segment. This suggests that the total numbers of T cell clonotypes responding to the two epitopes are similar.

In recent reports, the number of precursor CD8 T cells responsive to a single epitope was calculated to be 600 (46) or 3000 (47). From these calculations, it was not possible to determine how many of these clones contained unique CDR3. Our calculations, based on the number of different Vβ13 CD8 T cell clonotypes present in the CNS of infected mice, suggest that the number of different epitope S-510-518-specific T cell clonotypes is as high as 900 although this number is likely to underestimate the true diversity for the reasons described above.

Determination of the diversity of the CD8 T cell response is important in understanding the T cell response to specific Ags, including those present in vaccines. Direct sequencing of cDNA clones is most useful for evaluating the number of different T cell clones responding to an Ag and complements analyses that focus on the most abundant T cell clones in the immune response.

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