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Characterization of Mouse Hepatitis Virus-Specific Cytotoxic T Cells Derived from the Central Nervous System of Mice Infected with the JHM Strain

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The cytotoxic T lymphocyte (CTL) activity of spleen cells from BALB/c (*H-2*^{d}) mice immunized with the neurotropic JHM strain of mouse hepatitis virus (JHMV) was stimulated in vitro for 7 days. CTL were tested for recognition of target cells infected with either JHMV or vaccinia virus recombinants expressing the four virus structural proteins. Only target cells infected with either JHMV or the vaccinia virus recombinant expressing the JHMV nucleocapsid protein were recognized. Cytotoxic T cell lines were established by limiting dilution from the brains of mice undergoing acute demyelinating encephalomyelitis after infection with JHMV. Twenty of the 22 lines recognized JHMV-infected but not uninfected syngeneic target cells, indicating that they are specific for JHMV. All T-cell lines except one were CD8$^+$. The specificity of the CTL lines was examined by using target cells infected with vaccinia virus recombinants expressing the JHMV nucleocapsid, spike, membrane, and hemagglutinin-esterase structural proteins. Seventeen lines recognized target cells expressing the nucleocapsid protein. Three of the JHMV-specific T-cell lines were unable to recognize target cells expressing any of the JHMV structural proteins, indicating that they are specific for an epitope of a nonstructural protein(s) of JHMV. These data indicate that the nucleocapsid protein induces an immunodominant CTL response. However, no CTL activity specific for the nucleocapsid protein could be detected in either the spleens or cervical lymph nodes of mice 4, 5, 6, or 7 days after intracranial infection, suggesting that the CTL response to JHMV infection within the central nervous system may be induced or expanded locally.

Infection of rodents with the neurotropic JHM strain of mouse hepatitis virus (JHMV) results in a panencephalitis accompanied by extensive demyelination (14, 21, 25, 47, 49). Histological analyses of the central nervous system (CNS) show infiltration by mononuclear cells and primary demyelination (25, 49). The primary demyelination is characterized by macrophage-mediated removal of myelin and the preservation of axonal integrity. The mechanism of injury is cytolytic infection of oligodendroglial cells (25); however, an immunological component has recently been implicated (46).

The interaction of the immune response, the virus, and the cells of the CNS is not well understood. JHMV infection results in an acute phase characterized by viral growth prior to the recruitment of mononuclear cells into the CNS. Reductions in infectious virus are accompanied by a generalized panencephalitis and demyelination (25, 49). Survivors of subacute infections show evidence of chronic ongoing demyelination associated with the persistence of virus within the CNS (29, 42). Therefore, JHMV infection has been studied as a model for both the acute and chronic forms of virus-induced demyelination. The pathological changes in the CNS of rodents infected with JHMV have a number of features in common with multiple sclerosis, and indeed, recent evidence has suggested an association between coronaviruses and multiple sclerosis (28, 38).

To understand the relationships between JHMV, the CNS, and the immune response of the host, a number of studies have examined the ability of virus mutants to produce demyelination. These studies indicate that the spike (S) protein, which is the ligand for both neutralizing antibodies (2, 13, 48) and the cell surface receptor (10), plays a major role in the ability of JHMV to infect specific cell types within the CNS and produce demyelination (11, 14, 15). The role of the host immune response in suppressing virus replication, contributing to the characteristic virus-specific demyelination and resulting in viral persistence, is less clear. JHMV infection of immunologically compromised hosts indicates that immunity controls virus replication (3, 37, 43, 46) and, by inference, the ability of the virus to produce lesions within the CNS. Consistent with their role in controlling virus replication, neutralizing monoclonal antibodies (MAb) specific for the S protein protect against a lethal challenge (2, 48). Lethal infections can also be prevented by the passive transfer of MAb specific for nonneutralizing epitopes of the structural proteins; however, these MAb have little or no effect on virus replication or subsequent pathological changes within the CNS (12, 30, 56).

Analysis of the cells infiltrating the CNS during JHMV infection has shown that activated B cells, T cells, natural killer cells, and monocytes are all present within the CNS (9, 51, 53). In vivo depletion of T-cell subsets revealed that both CD4$^+$ and CD8$^+$ T cells are involved in the reduction of virus within the CNS and protection from acute disease (52). However, adoptive transfers have shown that virus clearance is associated with CD8$^+$ T cells restricted to major histocompatibility (MHC) class I molecules (44). These data were interpreted to indicate that a CD4$^+$ helper activity is required for the generation of cytotoxic CD8$^+$ T cells, which represent the primary effectors of JHMV clearance from the CNS (44). These data are also consistent with studies in which the

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adoptive transfer of virus-specific Th1 $CD4^+$ T cells were found to protect against lethal infection (41); however, more recent data have shown a partial reduction in virus titer in the CNS after adoptive transfer of Th1 clones (19, 55), suggesting that $CD4^+$ cells may also play a role in protection by suppressing viral replication. This could be accomplished via cytokine secretion or direct cytolysis of MHC class II-expressing cells within the CNS.

The data in this report demonstrate that both immunization and infection with JHMV induce a specific cytotoxic T-lymphocyte (CTL) response and that the only virus structural protein recognized is the N protein. Although the majority of the CTL lines isolated from the CNS during the acute phase of the infection are specific for the N protein, CTL specific for a nonstructural protein(s) were also isolated. No CTL activity specific for the N protein could be detected in either the spleen or cervical lymph nodes (CLN) at 4, 5, 6, or 7 days postinfection (p.i.) in a sensitive assay with target cells coated with a peptide containing the L^d-restricted N protein epitope. These data suggest preferential recruitment or local expansion of N protein-specific CTL within the CNS.

MATERIALS AND METHODS

Virus and cell lines. The neurotropic JHM strain of mouse hepatitis virus (JHMV) was propagated and plaque assayed with the murine DBT astrocytoma cell line as described previously (14, 39). The J774.1 ($H\text{-}2^d$) cell line was obtained from the American Type Culture Collection. The CV-1, $143Tk^-$, HeLa, and BSC-1 cell lines were obtained from B. Moss, Laboratory of Viral Diseases, National Institutes of Health.

Mice. BALB/c ($H\text{-}2^d$) mice were obtained from the Jackson Laboratory, Bar Harbor, Maine, and housed in isolator cages. Mice were immunized as described previously (40) with 10^6 PFU injected intraperitoneally. Mice were infected intracranially (i.c.) with 25 PFU of plaque-purified parental JHMV, designated DM, which is uniformly fatal within 7 to 9 days p.i.

Recombinant vaccinia viruses. The derivation of the vaccinia virus (VV) recombinants expressing the entire JHMV N protein (designated vJN); amino acids 301 to 455 of the JHMV N protein, which contains the L^d-restricted N protein CTL epitope (designated vJN51); and the control VV recombinant (vSC8), expressing the *Escherichia coli lacZ* gene, has been described previously (1, 40). The VV recombinant expressing the MHV-A59 S protein (8) was kindly provided by W. Spaan, University of Leiden, Leiden, The Netherlands.

The genes encoding the JHMV M protein (JM) and JHMV S protein (JS) were obtained by polymerase chain reaction (PCR) amplification of cDNA prepared from JHMV-infected J774.1 cells. Total mRNA was extracted from monolayers in 150-mm plates infected at a multiplicity of infection (MOI) of 1 at 12 h p.i. with guanidinium isothiocyanate. RNA was purified by centrifugation at 40,000 × *g* for 18 h through 5.7 M $CsCl_2$, resuspended in sterile water, and stored under ethanol in 30-μg aliquots at −20°C until use. M protein-specific cDNA was prepared from 10 μg of total mRNA with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, Ind.) and synthetic oligonucleotide primer SS11 (5′-AAA GGC CTT AGA TTC TCA ACA ATA CGG T; nucleotides [nt] 734 to 754), specific for the 3′ end of the M gene (32). The JM gene fragment was generated by PCR amplification of JM-specific cDNA (corresponding to 1 to 1.5 μg of reverse-transcribed mRNA) with synthetic oligonucleotide primers SS11 and SS12 (5′-TTG TCG ACA TGA GTA GTA CCA CTC AGG C; nt 68 to 87). Numbering of nucleotides includes 67 nt of untranslated leader sequence. *Sal*I and *Stu*I sites were incorporated into the primers to allow unidirectional cloning. After digestion with *Sal*I and *Stu*I, the 690-bp JM PCR fragment was subcloned into the *Sal*I and *Stu*I sites of plasmid pSC11ss (5).

The JS gene was constructed by sequential subcloning of 5′, middle, and 3′ gene fragments into plasmid pSC11ss as described below. Total RNA (15 μg) from JHMV-infected cells, treated with 1 μg of dactinomycin per ml throughout infection, was reverse transcribed with avian myeloblastosis virus reverse transcriptase and oligo$(dT)_{16}$ (Perkin-Elmer Cetus, Norwalk, Conn.) as a primer. All three JS gene fragments were generated by PCR amplification of cDNA with three pairs of S-specific oligonucleotide primers (45). Each fragment was designed to encode approximately one-third of the JS protein. Primers SS13 (5′-TTG TCG ACC ATG GTG TTC GTC TTT ATT TTA CTA TTA CC; nt 1 to 29) and TA72 (5′-CCC CAG GCC TCA AAT GGG GCC CAT TCC AAA; nt 1535 to 1548) were used to generate a 1,550-bp 5′ gene fragment incorporating sequences from the start codon to the *Apa*I site at position 1543. Numbering of nucleotides starts at the translation initiation codon ATG. 5′ *Sal*I-*Nco*I and 3′ *Apa*I-*Stu*I sites were introduced via the primers to allow unidirectional cloning into plasmid pSC11ss digested with *Sal*I and *Stu*I. The resulting plasmid, which contained coding sequences for the N-terminal 516 amino acids, was designated pSC1372. The middle gene fragment, extending from the *Apa*I (position 1543 in the S gene) to *Nru*I (position 2856) sites, was amplified with primers TA76 (5′-CCC CCC ATG GGG CCC CAT TTG CGC TCG GCA CGT; nt 1543 to 1566) and TA73 (5′ CCC CAG GCC TCG CGA ACT TCT TGA CCA CCA; nt 2841 to 2861). The resulting 1,320-bp fragment, containing terminal *Apa*I and *Stu*I sites, was digested with *Apa*I and *Stu*I and subcloned into the *Apa*I and *Stu*I sites of plasmid pSC1372 to yield plasmid pSC1373, encoding amino acids 1 to 953. Finally, the 3′ gene fragment, extending from the *Sph*I site (position 2508) to the end of the coding region (position 4128), was generated by PCR amplification with primers SM16 (5′-TTT TCC ATG GCA TGC AGA CAG CAG TTG G; nt 2508 to 2527) and TA125 (5′-CCC AGG CCT TCA ATC CTC ATG GGC TGA AAT A; nt 4110 to 4130), which contain *Sph*I and *Stu*I sites, respectively. After digestion with *Sph*I and *Stu*I, the 3′ 1,620-bp fragment was inserted into the *Sph*I and *Stu*I sites of plasmid pSC1373. Restriction analysis of the resulting plasmid, pSC125, confirmed that it contained the entire 4,127-bp coding region for the JS protein. The VV recombinant expressing the JHMV hemagglutinin-esterase (HE) gene was constructed by subcloning a 1.4-kb fragment encoding the HE gene derived by *Sal*I and *Stu*I digestion of plasmid pTZHE (57) into identical sites within pSC11ss.

The JHMV sequences were recombined into the wild-type WR strain of VV by lipofectin-mediated transfection of the plasmid DNA into WR-infected CV-1 cells. VV recombinants were plaque purified at least three times on $143Tk^-$ monolayers in the presence of 25 μg of bromodeoxyuridine per ml in the agarose overlay. Recombinant plaques were visualized with 300 μg of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside per ml as described previously (40). Virus pools used throughout were derived from crude cell lysates prepared from infected HeLa cells. Titers were determined by plaque assay on BSC-1 cell monolayers. Expression of the JHMV HE protein was confirmed by Western immunoblot with either HE-specific MAb or polyclonal anti-JHMV rabbit antiserum (56). Expression of the JHMV S and M proteins was confirmed by radioimmunoprecipitation from [^{35}S]methionine-labeled JHMV-infected DBT cells with MAb specific for the JHMV S

(J.2.2 and J.7.2) and M (J.1.3 and J.2.7) proteins as described previously (13).

Synthetic peptides and oligonucleotides. Peptide pN318-335, which contains the L^d-restricted N protein epitope (APTAGAFFFGSKLELVKK), was synthesized on an automated model 430A synthesizer (Applied Biosystems, Foster City, Calif.), and purity was assessed by high-pressure liquid chromatography (HPLC) as described previously (1). Oligonucleotides were synthesized on a model 394 DNA synthesizer (Applied Biosystems).

Induction of secondary CTL. Spleen cell suspensions were prepared from mice immunized 2 to 4 weeks earlier by intraperitoneal injection of 10^6 PFU of JHMV (40). Spleen cells from immune mice were cultured for 6 days at 37°C in 40 ml of RPMI 1640 medium supplemented with 2 mM glutamine, 25 μg of gentamicin per ml, 1 mM sodium pyruvate, 5×10^{-5} M β-2-mercaptoethanol, nonessential amino acids (RPMI complete), and 10% fetal calf serum (FCS; Gemini Bioproducts) with irradiated (2,500 rads) syngeneic spleen cells derived from naive mice which were infected with JHMV at an MOI of 0.10 to 0.05 as described previously (40).

Cytotoxicity assay. Spleen and cervical lymph node (CLN) cells from acutely infected mice, in vitro-stimulated spleen cells, and T-cell lines derived from the CNS of acutely infected mice were used as effector cells. Where indicated, spleen cells and CLN were depleted of B cells by panning with goat anti-mouse immunoglobulin (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). Effectors, in 100 μl of RPMI complete medium supplemented with 5% FCS, were added at various ratios to round-bottomed 96-well plates (Falcon Plastics, Oxnard, Calif.). J774.1 cells were infected with JHMV (MOI of 1 to 5) or VV recombinants (MOI of 5 to 10). After incubation for 6 h at 37°C, the target cells were washed twice in RPMI 1640, and 10^6 cells were labeled by incubation in 100 μCi of $Na^{51}CrO_4$ (New England Nuclear) in a volume of 100 μl for 1 h at 37°C, washed four times, and resuspended in RPMI 1640 containing 5% FCS. Target cells at a concentration of 10^4 in a 100-μl volume were added to the effector cells. In some experiments, target cells were preincubated for 30 min at 37°C with peptide (pN318-335), which has recently been shown to contain the immunodominant L^d-restricted N protein epitope (1). After 4 h of incubation at 37°C, 100 μl of supernatant was removed, and the radioactivity was determined in a gamma counter. Data are expressed as percent specific release, defined as (experimental release − spontaneous release)/(detergent release − spontaneous release). Maximum spontaneous release values were <20% of total release values.

Isolation of T-cell lines. To isolate cytotoxic T-cell lines from mice undergoing acute infection with JHMV, brains were removed at 7 days p.i. and pressed through 200-μm Nitex gauze (Tetko, Lancaster, Pa.) and single cells were isolated from the 30%–68% interface of Percoll step gradients as described previously (52). Viable cells were further isolated by centrifugation on Lympholyte M (Accurate Chemicals, San Diego, Calif.). After two washes in RPMI, the cells were suspended in RPMI complete containing 5% FCS, and adherent cells were removed by incubation on plastic petri dishes for 2 h at 37°C. Although adherence resulted in approximately 95% cell loss, T cells could not be expanded during four initial experiments in which this step was omitted. After vigorous washing, the nonadherent cells were recovered and incubated with syngeneic irradiated (2,500 rads) spleen cells infected with JHMV in Iscove's modified Dulbecco's modified Eagle's medium supplemented with 10% FCS and 10% rat concanavalin A supernatant containing 25 mM methyl-α-D-mannopyranoside as a source of growth factors. Cells were expanded by passage every 7 days in the same medium, beginning at the second week in culture, using JHMV-infected irradiated syngeneic spleen cells as feeders. $CD8^+$ T-cell lines were isolated from passage four after depletion of the $CD4^+$ T cells with rat MAb RL172.4 plus complement (40) by limiting dilution in 96-well plates containing 5×10^5 JHMV-infected feeder cells per well. Lines were expanded and maintained in RPMI complete medium supplemented with 10% FCS and 5 to 10% concanavalin A supernatant by weekly transfer of approximately 2×10^5 T cells per well of a 24-well plate containing 3×10^6 JHMV-infected irradiated syngeneic spleen cells.

FACS analysis. T-cell lines were examined for cell surface expression with rat anti-CD4 (MAb RL172.4), rat anti-CD8 (MAb 31M), or rat anti-T-cell receptor (TCR) vβ8.1-8.2 (MAb KJ-16) followed by fluorescein isothiocyanate-labeled goat anti-rat $(ab')_2$ antibody (Caltag, South San Francisco, Calif.) as described previously (53). Cells were labeled, washed, resuspended in phosphate-buffered saline containing 0.1% formaldehyde, and analyzed by fluorescence-activated cell sorting (FACS) on a FACStar (Becton Dickinson, Mountain View, Calif.).

RESULTS

Secondary BALB/c CTL recognize the JHMV nucleocapsid protein. Spleen cells obtained from mice immunized 3 to 4 weeks previously and stimulated in vitro for 7 days with JHMV-infected syngeneic spleen cells contain $CD8^+$ CTL which recognize target cells infected with JHMV (40). In addition, analysis of a VV recombinant expressing the N protein (vJN) demonstrated that the CTL activity specific for the N protein was restricted to the MHC class I L^d molecule (40). A panel of VV recombinants expressing truncations of the N protein showed that the N protein epitope was contained within the 149 carboxy-terminal amino acids. This sequence contains four potential L^d-restricted epitopes (6). Further analysis of this region with additional VV recombinants and synthetic peptides determined that the epitope encompasses amino acids 318 to 326 of the 455-amino-acid N protein (1).

To determine whether CTL specific for the other viral structural proteins were induced in mice of the $H\text{-}2^d$ haplotype, spleen cells from immunized mice were stimulated in vitro for 6 days with JHMV-infected irradiated syngeneic spleen cells and tested for recognition of target cells infected with JHMV or VV recombinants expressing the JHMV S, M, HE, and N structural proteins. Figure 1 shows that the cells present in these in vitro cultures only recognized target cells infected with JHMV and the VV recombinant (vJN) expressing the N protein. No cytotoxicity specific for the S, M, or HE protein could be detected in these populations. Figure 2 shows that the $CD4^-$ $CD8^+$ CTL in this population recognize target cells infected with JHMV and target cells expressing the endogenous N protein epitope (vJN51) as well as pN318-335 peptide-coated target cells. Furthermore, neither B-cell nor $CD4^+$ T-cell depletion influenced the recognition of target cells infected with JHMV or target cells expressing the N protein epitope (Fig. 2). Although the observed activity could be attributed solely to N protein-specific CTL, the 32-kb JHMV genome encodes a number of proteins within the remaining nonstructural coding sequence of approximately 23 kb (23, 24). Data presented below suggest that at least part of the anti-JHMV CTL activity is specific for an epitope(s) contained within a nonstructural protein(s).

CTL activity during acute infection with JHMV. To examine CTL activity during acute CNS infection with JHMV, spleen

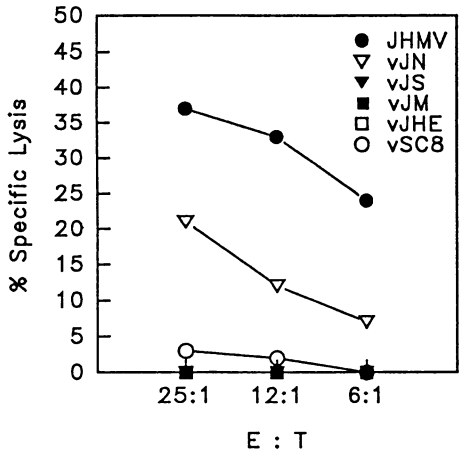

FIG. 1. Secondary CTL recognize JHMV-infected target cells and target cells expressing the JHMV N protein. Spleen cells from immunized BALB/c mice were cultured for 6 days in the presence of irradiated syngeneic feeder cells infected with JHMV and tested for recognition of J744.1 target cells infected with JHMV, VV recombinants expressing the JHMV S (vJS), M (vJM), HE (vJHE), and N (vJN) proteins, or the control VV recombinant vSC8.

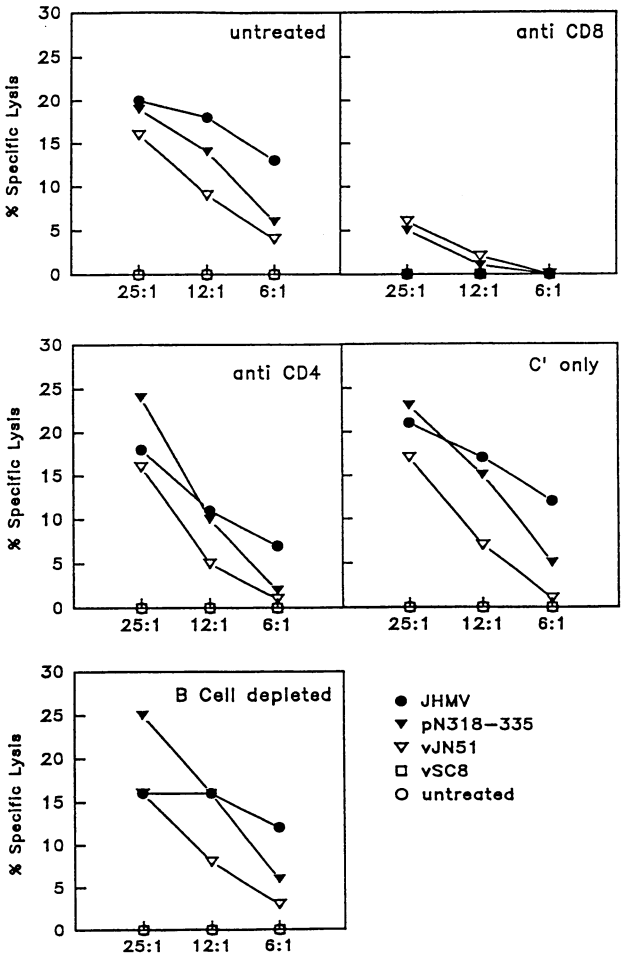

FIG. 2. Recognition of both exogenous and endogenously expressed N protein by secondary CTL. Spleen cells from immunized mice were cultured for 6 days in the presence of JHMV-infected syngeneic spleen cells. CTL activity was tested with untreated B-cell-depleted, CD8-depleted, CD4-depleted, and complement (C')-treated cells as indicated. The E/T ratio is shown on the *x* axis.

cells were obtained from mice infected i.c. 7 days previously. Initial experiments demonstrated significant cytolytic activity specific for target cells infected with either JHMV or a VV recombinant expressing the S protein derived from the heterologous strain MHV-A59 (vAS). No cytolysis specific for any JHMV-encoded structural protein, including the N protein, could be detected. The majority of the cytolytic activity specific for JHMV-infected target cells, but not the activity specific for the target cells expressing the MHV-A59 strain S protein, could be removed by treatment of the effector population with anti-CD8 MAb plus complement (not shown).

B-cell-mediated cytotoxicity of target cells expressing the MHV-A59 S protein has been described previously (50); therefore, effector populations were depleted of B cells by panning and examined for JHMV-specific cytotoxicity. Panning removed ≥90% of the B cells, as determined by FACS analysis, and eliminated the cytotoxicity specific for the MHV-A59 S protein. Figure 3 shows the cytolytic activity of the untreated and B-cell-depleted spleen cells on target cells infected with JHMV and VV recombinants expressing the JHMV S (vJS) and N (vJN) proteins and the MHV-A59 S protein (vAS). No cytotoxicity could be detected for target cells infected with VV recombinants expressing either the JHMV S or N protein (Fig. 3) or the JHMV M or HE protein (data not shown). These data confirm the previous demonstration of B-cell-mediated cytolysis of MHV-A59-infected target cells (50, 54) but suggest that neither the target cells infected with JHMV nor the target cells expressing the JHMV S protein are susceptible to B-cell-mediated cytolysis. Furthermore, these data suggest that CTL specific for a viral nonstructural protein were induced following i.c. infection with JHMV and that this population mediates the dominant response detected in the spleen of mice undergoing an acute infection with JHMV.

Isolation and characterization of CTL from the CNS. Mononuclear cells were isolated from the brains of mice infected i.c. 7 days earlier with 25 PFU of JHMV as described previously (53) and allowed to adhere to plastic dishes for 2 h at 37°C. Proliferating T cells were depleted of $CD4^+$ cells by treatment with MAb plus complement (40), and the viable cells were cloned at limiting dilution. Twenty-two cell lines were isolated and tested for recognition of JHMV-infected and uninfected J774.1 target cells. Figure 4 shows that two lines, designated B1 and B2, could not distinguish infected from uninfected target cells, consistent with the activation of self-reactive T cells after JHMV infection (22). The remaining 20 T-cell lines showed various levels of specific lysis of JHMV-infected target cells.

To determine which viral proteins were recognized by the JHMV-specific CTL lines, target cells infected with VV recombinants expressing the JHMV S, M, HE, and N structural proteins were tested for recognition at either two or three effector-to-target (E/T) ratios. In some experiments, the recently identified L^d-restricted N protein-derived peptide pN318-335 (1) was used as an antigen in place of the N protein expressed by the vJN recombinant VV. Target cells infected with the VV recombinant expressing the N protein (vJN) are recognized less efficiently than those infected with two other VV recombinants expressing either a carboxy-truncated form of the N protein (vJNb) or the internal 51 amino acids (vJN51)

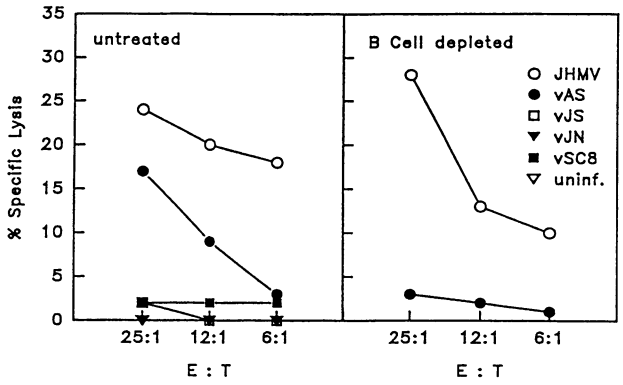

FIG. 3. CTL activity in the spleen of BALB/c mice 7 days after i.c. infection with JHMV. CTL activity of untreated and B-cell-depleted spleen cells from mice infected 7 days before the assay was determined. Target cells were infected with JHMV or VV recombinants expressing the MHV-A59 S protein (vAS), the JHMV S protein (vJS), or the JHMV N protein (vJN).

containing the CTL epitope, possibly due to the presence of 3′ untranslated sequences (1, 40). In addition, target cells coated with the pN318-335 peptide are more sensitive to CTL recognition than any of the VV recombinant-infected cells (1).

Examples of the recognition patterns obtained for JHMV- and VV recombinant-infected target cells are shown in Table 1, and the complete characterization of all 22 lines is summarized in Table 2. As for JHMV-immunized mice, no response was detected for any of the structural proteins other than the N protein. A total of 17 CTL lines were specific for target cells expressing the N protein. Three lines, designated B14, B18, and B22, recognize JHMV-infected target cells. These lines did not recognize target cells infected with VV recombinants expressing any of the structural proteins or target cells coated with the pN318-355 peptide, suggesting that they are specific for a nonstructural protein(s). Therefore, the majority (17) of

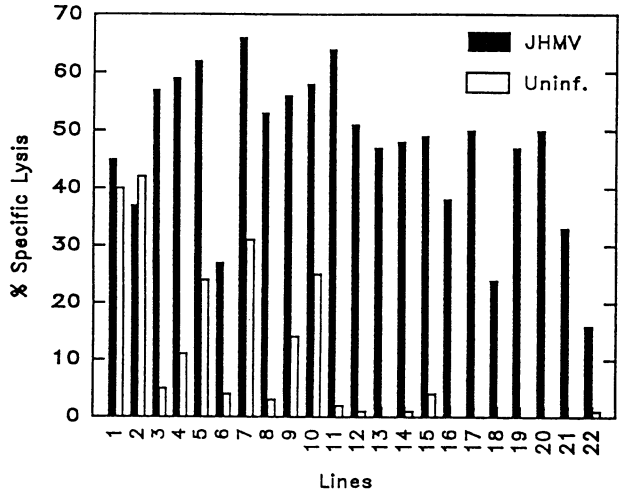

FIG. 4. JHMV-specific CTL lines cultured from the CNS of JHMV-infected mice. CTL activity was tested at a single E/T ratio of 2 to 5 for each line with JHMV-infected and uninfected J774.1 target cells.

TABLE 1. Specificity of brain-derived T-cell lines

CTL line	E/T ratio	Specific ^{51}Cr release by target cells[a]						Protein specificity
		JHMV	M	S	HE	N	Control	
B3	5:1	NT[b]	3	3	4	**13**	2	N
	2.5:1	NT	2	2	2	**9**	1	
B5	10:1	30	0	0	4	**15**	4	N
	5:1	20	0	0	1	**9**	5	
B6	10:1	27	0	0	1	**24**	4	N
	5:1	22	0	0	2	**21**	3	
B8	8:1	NT	2	3	3	**28**	2	N
	4:1	NT	1	0	0	**19**	0	
B10	10:1	29	0	0	0	**19**	0	N
	5:1	16	0	0	0	**15**	0	
B11	10:1	NT	5	1	1	**42**	1	N
	5:1	NT	2	0	1	**30**	0	
B14	5:1	**43**	8	0	0	0	8	Nonstructural
	2.5:1	**34**	7	0	0	0	7	
B17	6:1	NT	0	0	1	**17**	0	N
	3:1	NT	0	0	1	**14**	0	
B22	10:1	**21**	0	0	NT	3	0	Nonstructural
	5:1	**11**	0	0	NT	4	0	

[a] Target cells were infected with JHMV or with VV recombinants expressing the JHMV M, S, HE, or N protein. The control was J774.1 cells infected with vSC8 or uninfected cells.
[b] NT, not tested.

the T-cell lines isolated from the CNS of acutely infected mice are specific for the N protein.

Flow cytometry analysis of the CTL lines showed that all of the cytolytic lines, including the two nonspecific lines B1 and B2, were $CD8^+$, with one exception (B22). The N protein-specific CTL response in BALB/c mice is exclusively L^d restricted (40); therefore, these data indicate that the L^d-restricted response to the N protein is the immunodominant CTL response in the CNS of BALB/c mice undergoing acute JHMV infection. In addition, these data confirm the presence of a CTL response directed toward an epitope(s) contained within a nonstructural protein(s).

One cytolytic $CD4^+$ T-cell line (B22) was also isolated from the CNS of JHMV-infected mice (Tables 1 and 2). The B22 line exhibits a unique dual specificity of recognition. It recognizes target cells infected with JHMV (Table 1, Fig. 4) and with a VV recombinant expressing the MHV-A59 S protein (vAS) but not target cells infected with the VV recombinant expressing the JHMV S protein (vJS) (Fig. 5). However, the cytolytic activity specific for the MHV-A59 S protein is not MHC class I restricted (data not shown), suggesting that this $CD4^+$ line mediates lysis of the S protein-expressing target cells via interaction between the MHV-A59 S protein and its cell surface receptor. The recognition of the JHMV-infected targets but not of target cells infected with any of the VV recombinants expressing the virus structural proteins suggests that B22 recognizes an epitope encoded within a nonstructural protein. Neither of the other two $CD8^+$ T cell lines (B14 and B18), which recognize an epitope within a nonstructural protein(s), recognized target cells expressing the MHV-A59 S protein.

The different growth rates of the isolated lines propagated

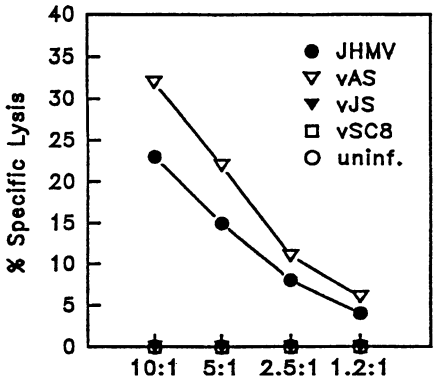

FIG. 5. Cytolytic activity of the CD4+ B22 T-cell line. B22 was tested for recognition of J774.1 target cells infected with JHMV or VV recombinants expressing the JHMV S protein (vJS), the MHV-A59 S protein (vAS), and the control VV recombinant expressing the *E. coli lacZ* gene (vSC8).

under identical conditions suggested that they were not derived from a single precursor. To examine the high proportion of the JHMV N protein-specific CTL lines isolated, we first determined the number of N protein-specific T-cell lines which express the vβ8.1/8.2 TCR with MAb KJ-16 (17). Increased frequency of vβ8 use has been associated with L^d-restricted CTL responses (16, 36), and the CTL response to the N protein is exclusively L^d restricted (40). The data in Table 2 show that 10 of the 15 N protein-specific CTL lines did indeed express vβ8.1/8.2 TCR. Two lines were unavailable for testing (B5 and B10), and one line (B3) was determined by FACS analysis not to be clonal, containing both KJ-16+ and KJ-16− CD8+ T cells. Analysis of the three lines specific for a

TABLE 2. Characteristics of JHMV-specific CNS-derived CTL lines

Line	Protein specificity[a]	Phenotype[b]	KJ-16 expression
B1	Nonspecific	CD8+	NT[c]
B2	Nonspecific	CD8+	NT
B3	Nucleocapsid	CD8+	+
B4	Nucleocapsid	CD8+	−
B5	Nucleocapsid	NT	NT
B6	Nucleocapsid	CD8+	−
B7	Nucleocapsid	CD8+	+
B8	Nucleocapsid	CD8+	+
B9	Nucleocapsid	CD8+	−
B10	Nucleocapsid	NT	NT
B11	Nucleocapsid	CD8+	+
B12	Nucleocapsid	CD8+	−
B13	Nucleocapsid	CD8+	+
B14	Nonstructural	CD8+	−
B15	Nucleocapsid	CD8+	+
B16	Nucleocapsid	CD8+	+
B17	Nucleocapsid	CD8+	+
B18	Nonstructural	CD8+	+
B19	Nucleocapsid	CD8+	−
B20	Nucleocapsid	CD8+	+
B21	Nucleocapsid	CD8+	+
B22	Nonstructural	CD4+	−

[a] Specificity was tested with J774.1 target cells infected with VV recombinants expressing the JHMV S, M, N, and HE proteins or target cells coated with the pN318-335 peptide.
[b] Phenotype was determined by flow cytometry.
[c] NT, not tested.

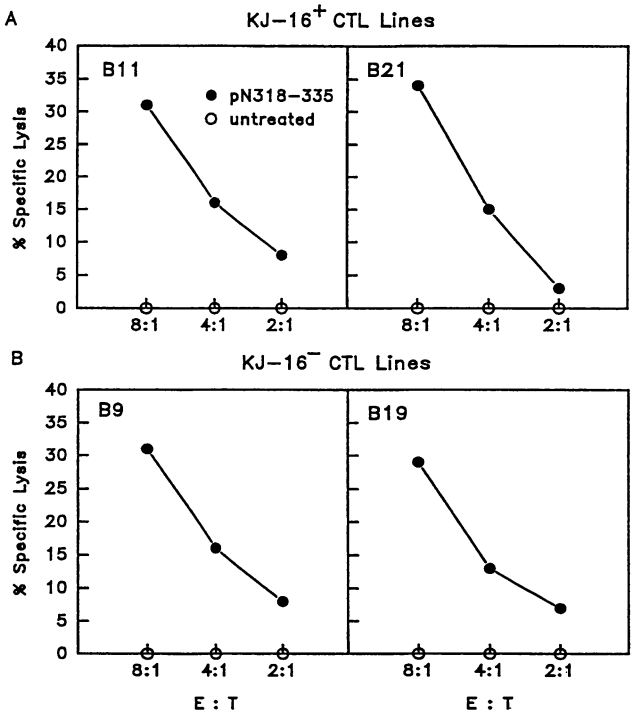

FIG. 6. Recognition of pN318-335 by KJ-16+ and KJ-16− CTL lines. (A) Recognition of the pN318-335 peptide on J774.1 target cells by KJ-16+ CTL lines B11 and B21. (B) Recognition of pN318-335 peptide on J774.1 target cells by KJ-16− CTL lines B9 and B19.

nonstructural protein epitope(s) showed that only one line (B18) expressed the vβ8.1/8.2 TCR (Table 2).

Our previous data demonstrated that the KJ-16+ CTL lines B8 and B15 recognized peptide pN318-335, derived from the N protein, on L^d-expressing target cells (1). The N protein contains a number of potential L^d-binding motifs (6). To determine whether the KJ-16+ and KJ-16− CTL lines had the same specificity, two additional KJ-16+ CTL lines (B11 and B21) and two KJ-16− lines (B9 and B19) were tested for recognition of J774.1 target cells coated with the pN318-335 peptide. Figure 6 shows that these four CTL lines recognize target cells incubated in the presence of half-saturating concentrations of peptide pN318-335 (5 nM). These data suggest that a single N protein-derived L^d-restricted epitope is recognized by both KJ-16+ and KJ-16− CTL lines.

Absence of N protein-specific CTL in the CLN populations. To determine whether CTL specific for the N protein were present in the CLN, cells were isolated from the CLN of mice 7 days after i.c. infection with JHMV and tested directly for cytotoxic activity. Target cells infected with JHMV, VV recombinants expressing the MHV-A59 S protein (vAS) or the JHMV S protein (vJS), and target cells incubated with pN318-355 were examined. Figure 7A demonstrates the presence of cytolytic activity in the CLN cell population specific for JHMV-infected and MHV-A59 S protein-expressing target cells. Depletion of B cells eliminated the cytolytic activity specific for the MHV-A59 S protein but not the CTL activity specific for the target cells infected with JHMV (Fig. 7). Depletion of CD8+ cells eliminated all JHMV-specific cytotoxicity (data not shown). As for the populations derived from the spleens of infected mice, no N protein- or S protein-specific CTL activity could be detected in the CLN population. In addition, no CTL

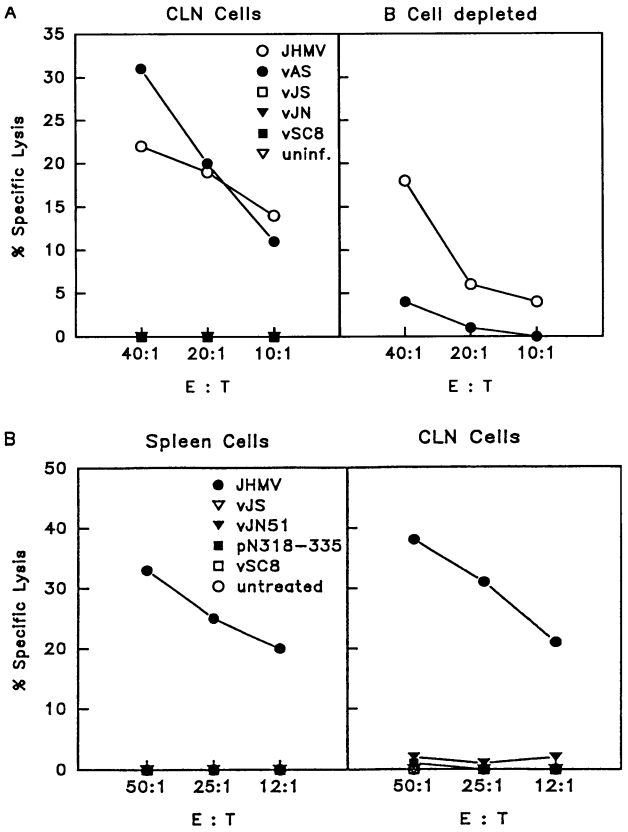

FIG. 7. CTL activity of CLN and spleen cells from JHMV-infected mice. (A) Cytolytic activity of untreated or B-cell-depleted CLN cells obtained 7 days p.i. and tested on target cells infected with JHMV or VV recombinants expressing the JHMV S (vJS) and N (vJN) proteins or the MHV-A59 S protein (vAS) compared with uninfected and vSC8-infected target cells. (B) CTL activity in spleen cells and CLN cells 7 days p.i. for recognition of target cells infected with JHMV, vJS, vJN51, or vSC8, untreated cells, and target cells coated with the pN318-335 peptide.

activity specific for targets expressing the JHMV M or HE protein was detected (data not shown).

The possibility that the CTL specific for the N protein in either the spleen or CLN populations were rapidly recruited to the CNS was examined by analysis of the spleen and CLN populations for N protein-specific CTL at 7, 6, 5, and 4 days p.i. Figure 7B shows that neither the spleen nor the CLN cells from mice infected 7 days previously recognize target cells either infected with the VV recombinant expressing amino acids 301 to 351 of the N protein (vJN51) or incubated with 100 nM pN318-335 peptide. Identical results were obtained from parallel analysis of spleen and CLN cells from mice infected 6, 5, and 4 days previously (data not shown). These data suggest that the N protein-specific CTL either are generated locally within the CNS or are present at extremely low levels within the peripheral lymphoid organs and recruited rapidly into the CNS.

DISCUSSION

Manipulations of the immune response of rodents infected with JHMV have shown both beneficial and detrimental effects (21). The passive transfer of nonneutralizing MAb specific for structural proteins and the adoptive transfer of virus-specific $CD4^+$ T cells are both able to prevent death from lethal infections. However, neither of these manipulations result in a significant alteration of virus replication within the CNS. The in vivo depletion of T-cell subsets during JHMV infection suggested that the initial clearance of virus from the CNS is mediated by a $CD4^-$ $CD8^-$ population (52). Natural killer cells were further implicated in this initial clearance by direct analysis of natural killer activity in mononuclear cell infiltrates obtained during the acute infection (53). However, these studies also clearly showed that subsequent clearance of virus from the CNS requires both $CD4^+$ and $CD8^+$ T-cell populations (52), consistent with the suggestion that induction of JHMV-specific CTL requires a $CD4^+$ "helper" population (44). Finally, the adoptive transfer of clonal populations of JHMV-specific $CD8^+$ T cells is of therapeutic value, resulting in both protection from a lethal challenge and suppression of virus replication in the CNS (55), consistent with the hypothesis that $CD8^+$ T cells play a major role in protection from JHMV-induced disease (44, 52).

The present study demonstrates that the CTL response specific for the N protein constitutes a major JHMV-specific effector CTL population present both in mice immunized intraperitoneally and within the CNS of infected mice. The majority of the CTL isolated from the CNS of mice infected with JHMV are specific for the N protein and express the Vβ 8.1/8.2 TCR. Although it is difficult without further analysis to determine whether these T cell lines are indeed unique, a very high proportion of L^d-restricted T cells show preferential vβ8 use (16, 36). Similarly, the low frequency of $v\beta8.1/8.2^-$ clones could also reflect the isolation and expansion of sister clones. Nevertheless, these data clearly demonstrate that CTL specific for a single virus structural protein infiltrate the CNS during acute JHMV infection.

Consistent with these results, it has recently been reported that N protein-specific CTL can be demonstrated in both the brain and spinal cords of BALB/c mice infected with JHMV as early as 7 days p.i. (4). All eight of the N protein-specific CTL analyzed, comprising both $v\beta8.1/8.2^+$ and $v\beta8.1/8.2^-$ T-cell lines, recognize targets coated with the pN318-335 peptide (Table 2) (1). These data further indicate that the L^d-restricted N protein epitope (amino acids 318 to 326) represents the immunodominant epitope recognized within the CNS of infected mice. Analysis of the clonal populations derived from the CNS of JHMV-infected mice also suggests the presence of at least one additional JHMV epitope recognized within the context of the $H\text{-}2^d$ haplotype that is presumably encoded within a nonstructural protein(s). These data contrast with the findings that the CTL specific for JHMV found in the CLN and spleen populations during the acute infection of the CNS are not specific for the N protein but are presumably specific for a nonstructural protein(s).

It is somewhat surprising that no $H\text{-}2^d$-restricted epitopes were detected within the three other virus structural proteins examined. However, only a single L^d-restricted epitope was detected within the N protein (1) despite the presence of at least eight additional amino acid sequences which conform to the recently described L^d epitope motif (6). Although CNS cells in vitro express MHC-derived gene products, there is evidence that cells within the adult CNS are unable to express either class I or class II MHC molecules (26). This could be interpreted to suggest that the clearance of JHMV from the CNS in mice protected by $CD8^+$ T cells is due primarily to cytolytic activity directed toward infected microglia or macrophages recruited during encephalomyelitis or via the local release of lymphokines. However, the growth of at least one JHMV variant predominantly in oligodendroglial cells and its

clearance from the CNS with approximately the same kinetics as wild-type virus (15) suggest that virus clearance is mediated by lymphokines, for example, gamma interferon, rather than by direct lysis of virus-infected cells (20). This concept is consistent with the recent demonstration that a VV recombinant expressing gamma interferon is cleared more rapidly than parental virus (34). Alternatively, the recent demonstration of MHC class II expression on oligodendrocytes in vivo during Theiler's virus infection (33) suggests that JHMV infection might also induce MHC gene expression in vivo, facilitating virus clearance from the CNS via direct cytolysis of infected CNS cells. Consistent with this, Yamaguchi et al. (55) reported increased cytopathology within the CNS of mice protected by adoptive transfer of JHMV-specific $CD8^+$ CTL clones.

B cells from naive BALB/c mice exhibit cytotoxicity for cells infected with MHV-A59, resulting in death of both the target and the effector B cell (50). This cytolytic activity can be blocked by antibody specific for the S protein, suggesting that the cytolysis is mediated by the interaction of the MHV-A59 S protein with a surface component expressed by the B cells. Recent data suggest that this cytolytic interaction is mediated by the interaction between the S protein and its ligand, the MHV receptor (10), on the B-cell population (18). The data in the present report suggest that B-cell-mediated killing does not contribute significantly to the cytolytic activity directed against JHMV-infected targets, since the JHMV-specific cytolytic activity was not removed by B-cell depletion. Furthermore, our data suggest that some $CD4^+$ T cells may also express the MHV receptor. One $v\beta8.1/8.2^-$ $CD4^+$ cytolytic clone (B22) was isolated which recognizes JHMV-infected but not uninfected targets, demonstrating specificity for a viral component. This line is also able to lyse target cells expressing the MHV-A59 S protein but not target cells expressing the JHMV S protein. Although the coding sequence of the JHMV S protein within VV recombinant vJS has not been completely determined, cell lines expressing the MHV receptor but not receptor-negative cells are fused after infection with vJS, indicating that the S protein expressed by vJS is able to interact with the MHV receptor.

The ability of B cells from BALB/c mice and the B22 clone to lyse targets infected with a VV recombinant expressing the MHV-A59 but not the JHMV S protein suggests quantitative differences between the interactions of these two viral glycoproteins and their cellular receptor. Furthermore, the in vivo depletion of T-cell subsets demonstrated that both $CD4^+$ and $CD8^+$ T-cell populations were required for the effective clearance of virus from the CNS (52). These data were interpreted to indicate that induction of the $CD8^+$ CTL required $CD4^+$ helper activity (44, 52). Although the present data do not contradict this hypothesis, they suggest that virus-specific $CD4^+$ cytolytic T cells may contribute directly to the reduction of JHMV during infection within the CNS.

The absence of detectable N protein-specific CTL in both the spleen and CLN during JHMV infection may provide new insights into the interactions between the immune system and the CNS. N protein-specific CTL could not be detected in the spleen or CNL from days 4 to 7 p.i., even in the extremely sensitive peptide-coated target assay system (1). These data suggest either that the number of N protein-specific CTL in the spleen and CLN is too low to be detected or that these CTL are rapidly recruited and expanded within the CNS. The rapid recruitment of antigen-specific T cells into the CNS and their subsequent expansion may simply reflect the large quantity of N protein produced. Alternatively, the unique nature of the infection may result in a localized induction and expansion of these cells.

Local expansion of defined T-cell subsets has previously been postulated to account for the limited TCR diversity detected in the CNS of patients with multiple sclerosis (31). Recent evidence has suggested that astrocytes can prime $CD8^+$ but not $CD4^+$ T cells (35), supporting the possibility of local expansion of N protein-specific $CD8^+$ CTL. Previous studies, using either injection of soluble proteins (7) or viral infections (27) which result in a predominant meningitis, have implicated the CLN as the major draining lymph nodes for the CNS. By contrast, JHMV produces a predominant encephalomyelitis associated with virus replication in oligodendroglia and astrocytes, with only minimal meningitis (25, 49).

Whether the site of virus replication or the cell types infected influence the CTL component of the immune response is not known; however, proliferative responses to JHMV within the CLN can be detected at least as early as 5 days p.i. (51). These data show the presence of JHMV-specific CTL which do not recognize any of the individual viral structural proteins in the spleen and CLN. This finding is not surprising, given the large proportion of the MHV genome encoding nonstructural proteins (approximately 23 of the 32 kb total). However, the low frequency of $CD8^+$ CTL lines specific for a nonstructural protein(s) (2 of 19) suggests that these cells are not efficiently recruited into the CNS during JHMV infection and may reflect differences in adhesion molecule expression.

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