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Mouse Hepatitis Virus Nucleocapsid Protein-Specific Cytotoxic T Lymphocytes Are Ld Restricted and Specific for the Carboxy Terminus

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Infection of mice with the JHM strain of mouse hepatitis virus (MHV) results in an acute encephalomyelitis associated with primary demyelination of the central nervous system. Efforts at understanding the components of the immune response in the development of chronic MHV-induced demyelination have implicated the antibody response and both the CD4+ and CD8+ T cell responses. In this report, we demonstrate that Balb/c (H-2b) mice immunized with the JHM virus (JHMV) strain of MHV develop a CD8+ cytotoxic T lymphocyte (CTL) response. One population of these virus-specific CTL recognize the nucleocapsid (N) protein. Recombinant vaccinia viruses expressing either the entire N protein or carboxy-terminal deletions were used to determine the number and location of the epitope(s) recognized. The CTLs were found to recognize a peptide contained within the carboxy-terminal 149 amino acids of the N protein. Analysis of infected cell lines expressing transfected major histocompatibility genes demonstrated that the anti-N protein CTLs were restricted exclusively to the Ld molecule. These data provide the first definition of a MHV-specific CTL response directed to a viral protein and suggest that the anti-N protein CTL response is one potential mechanism used by the host to clear JHMV from the central nervous system.

INTRODUCTION

Coronaviruses are enveloped, plus-stranded RNA viruses containing a genome of approximately 31 kb and three or four structural proteins (Lai, 1990). The murine coronavirus, mouse hepatitis virus (MHV), produces a variety of acute and chronic infections in its natural host, the mouse. The first isolate, designated JHM virus (JHMV), was derived from a single mouse with hind leg paralysis (Cheever et al., 1949). Interest in the pathogenesis of these viruses has centered on their ability to produce both acute and chronic central nervous system (CNS) demyelination in mice (Weiner, 1973; Stohlman and Weiner, 1981; Kyuwa and Stohlman, 1990). Immunosuppression of JHMV-infected mice results in an acute fulminant encephalomyelitis with little or no evidence of demyelination, indicating that the immune response plays a critical role in pathogenesis (Williamson and Stohlman, 1990). The passive transfer of monoclonal antibodies (mAb) specific for each of the four structural proteins provides protection from the acute phase of the disease; however, they appear to exacerbate the chronic form of the disease (Buchmeier et al., 1984; Wege et al., 1984; Fleming et al., 1988, Yukumori, unpublished data). Similarly, the adoptive transfer of MHV-specific CD4+ delayed-type hypersensitivity (DTH)-inducer Th1 T cells prevents the initial acute form of the disease (Stohlman et al., 1986). The immune response in the CNS induced by the transfer of these cells appears to resemble a typical DTH response; however, similar to the passive transfer of anti-viral mAb, no reduction in virus titer was found in the recipients. In addition, CD4+ T cell-mediated protection also exacerbated the late form of the disease. Nonviral-specific DTH responses did not similarly affect the course of the disease (Stohlman et al., 1988), suggesting the requirement for a viral-specific component. A common theme in these studies is that animals protected from the acute disease, in which virus replication is not suppressed, appear to be more susceptible to the chronic form of JHMV-induced CNS disease. Cytotoxic T lymphocytes (CTL) are an important component of the immune response to many viral infections. CTL have been implicated in the pathogenesis of MHV infection by the demonstration that a reduction in MHV titer during infection of C57Bl/6 mice requires a CD8+ T cell (Sussman et al., 1987, 1989). Further, the clearance of virus requires compatibility between donor and recipient at the major histocompatibility complex (MHC) Class I genes. These data also indicated that a CD4+ helper T cell is required for the activation of the CD8+ effectors (Sussman et al., 1989). This dependence on CD4+ T cells has been confirmed by the in vivo depletion of CD4+ and CD8+ T cells (Williamson et al., 1988).
The CV-1, 143TK-, and BSC-1 cell lines were obtained from Dr. B. Moss, Laboratory of Viral Diseases, NIAID. The J774.1 (H-2d) cell line was obtained from ATCC.

Virus, vSC8, created by recombining the pSC-1 l,,... sons Laboratories (Bar Harbor, MA) at 6 weeks of age. Mouse strains and cell lines

Mouse strains and cell lines

Balb/cBy (H-2b) mice were purchased from the Jackson Laboratories (Bar Harbor, MA) at 6 weeks of age. The J774.1 (H-2b) cell line was obtained from ATCC. The CV-1, 143TK-, and BSC-1 cell lines were obtained from Dr. B. Moss, Laboratory of Viral Diseases, NIAID. L929 cell (H-2k) derivatives designated K830 (expressing the Nd gene), K2A7 (expressing the Ld gene), and H40.5 (expressing the Kd gene) (Goodenow et al., 1982; Stroynowski et al., 1985) were obtained from Dr. M. McMillan, University of Southern California.

Generation of recombinant vaccinia viruses

The JHMV N gene coding sequence was amplified by PCR from a cDNA encoding the JHMV N protein contained in a pT7-3 plasmid (supplied by Dr. M. M. C. Lai, University of Southern California). The oligonucleotides IF-5 (5'-GGGGGGTCGACCTTTAAGGGATGCTTTTTGTTTCCCTGGGC-3') and IR-5 (5'-GGGGAGCCCTTCTGAGATGTAGTCGCCGGAGGGG-3'), which contain Sad and Sful sites, respectively, were used for PCR amplification. The PCR product was digested with Sad and Sful and ligated into similarly digested vaccinia virus shuttle vector pSC-11ss (supplied by Dr. B. Moss, Laboratory of Viral Diseases, NIAID). The resulting construct, pSC-11N, which contains the entire N gene coding sequence, was used for the generation of all subsequent deletion constructs. Deletions were made by digestion with NruI (located 41 nt 3' of the N gene stop codon) and Apal (nt 402), Nael (nt 746), or Spel (nt 915) which are contained within the N gene coding sequence. Termini were blunt-ended and the plasmids were religated, generating N gene truncations while also creating new, in-frame stop codons. The plasmid constructs also contain three stop codons immediately 3’ of the N gene in addition to the newly created stop codons. The deletion constructs, including both the initiation and stop codons, were verified by sequence analysis using the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical) and two flanking pSC 11-specific oligonucleotides: pSC11L (5'-CCAAACCCACCCGCTTTTATAG 3') and pSC11R (5'-CGTTGAATGTCCATGGAGTCGGGC-3'). The constructions were then introduced into the WR strain of vaccinia virus by homologous recombination in CV-1 cells (Mackott et al., 1984). Recombinant vaccinia viruses were selected by three or four plaque purifications on 143 Tk+ cells using 25 μg/ml bromodeoxyuridine in the initial agarose overlay. Recombinant plaques were visualized using 300 μg/ml of 5-bromo-4-chloro-3 indolyl-b-d-galactopyranoside (X-Gal) in a second agarose overlay as described by Chakrabarti et al. (1985). Stocks were prepared by propagation of the plaque isolates in CV-1 cells followed by expansion in HeLa cells (Mackett et al., 1984). Virus titers were determined by plaque assay on BSC-1 cells (Mackett et al., 1984). The recombinant vaccinia virus expressing the full-length N protein was designed VVIn, while those expressing the carboxy-terminal deletions were designed VVInns for the Nael deletion, VVInn for the NaeI deletion, and VVInNa for the Apal deletion.

Western blot analysis

DBT cells were infected with vSC8 (containing no insert), or recombinant vaccinia viruses containing ei-
ther the full-length or truncated N gene sequences. At 24 hr postinfection, the cells were washed 1× in PBS and lysed in Laemmli sample buffer (Laemmli, 1970). The lysates were subjected to electrophoresis in 12% SDS–polyacrylamide gels and transferred to nitrocellulose. The expression of N gene products was detected by Western blot using a mixture of anti-JHMV N protein-specific mAbs (Fleming et al., 1983), followed by visualization with ECL reagents (Amersham).

**Induction of bulk effector CTL**

Spleen cell suspensions were prepared from mice primed 3–8 weeks earlier by intraperitoneal (ip) injection of either 1 × 10^6 PFU of the DL isolate of JHMV (Stohlman et al., 1982) or 5 × 10^7 PFU of the recombinant vaccinia virus VVJN. Spleen cells (1 × 10^8) from immune mice were cultured for 6 days at 37°C with 5 × 10^7 irradiated (2500R) syngeneic spleen cells from naive mice infected with either JHMV [multiplicity of infection (m.o.i.) of 0.06] or VVJN (m.o.i. of 5.0) in 10 ml of RPMI 1640 medium supplemented with 10% fetal calf serum (Gemini Bioproducts), 2 mM glutamine, 25 µg/ml gentamycin, 1 mM sodium pyruvate, 5 × 10^-5 M β-2-mercaptoethanol, and nonessential amino acids (RPMI-10).

**Cytotoxicity assay**

*In vitro* stimulated spleen cells in 100 µl of RPMI-10 were added at various ratios to round-bottom 96-well plates (Falcon Plastics) containing 1 × 10^4 target cells in 100 µl of RPMI-10. J774.1 cells or the various L929 cell transfectants expressing the individual MHC H-2d genes were used as targets. J774.1 cells were infected with JHMV or recombinant vaccinia viruses at an m.o.i. of 5 to 10. The L929-derived cell lines were infected with recombinant vaccinia viruses at an m.o.i. of 50. Following incubation for 6 hr at 37°C the targets were washed 2× in RPMI and 1 × 10^6 cells were labeled with 100 µCi Na^85^CrO_4_ (New England Nuclear) in a volume of 100–200 µl, washed 4× with RPMI, resuspended in RPMI-10, and added to the effector cells. After 4 hr incubation at 37°C, 100 µl of the supernatant was removed and the radioactivity determined in a gamma counter. Data are expressed as percentage specific release defined as ([experimental release] – [spontaneous release])/[Total (detergent release) – [spontaneous release]]. Maximum spontaneous release values were always <20% of total release values.

**Complement-mediating depletion**

Secondary effectors derived from JHMV-immunized mice were cultured *in vitro* for 6 days with irradiated (2500 R) syngeneic splenocytes infected with VVJN at an m.o.i. of 5 to 10. The cells were washed twice by centrifugation in RPMI, resuspended in 2.0 ml RPMI at 2 × 10^6 viable cells per milliliter, and incubated for 1 hr at 4°C either with mAb specific for CD8 (Sarimento et al., 1980), CD4 (Ceredig et al., 1985), or without antibody. The cells were washed twice in RPMI, resuspended in 1.8 ml RPMI containing 25 mM HEPES, pH 7.2, and 0.3% bovine serum albumin. Low-Tox Rabbit complement (Cedarlane) was added at 0.2 ml and the mixture incubated 45 min at 37°C. Following two additional washes, the cells were resuspended and tested for residual CTL activity.

**RESULTS**

**Construction and expression of N protein deletions in vaccinia virus**

DNA encoding the N protein of JHMV was amplified by PCR using the primers IF-5 and IR-5 and cloned into the SalI and StuI sites of pSC 118, as described under Materials and Methods. The resulting construct was designated pSC-11N and was used for all subsequent N gene deletions. The deletions ranged from the ApaI, SpeI, or Nael sites within the coding sequence and the unique NruI site in the 3'-untranslated region and followed by ligation (Materials and Methods). These plasmids were designated pSC-11Na, pSC-11Nn, and pSC-11Ns, respectively. Each plasmid was used to construct a recombinant vaccinia virus by homologous recombination and the viruses derived from each were designated VVJN, VVJNs, VVJNn, and VVJNa.
FIG. 2. N protein expression by recombinant vaccinia virus. Lysates of recombinant vaccinia virus-infected DBT cells were prepared at 24 hr postinfection. Proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose. The blot was probed with a pool of mAb specific for the N protein obtained from Balb/c mice immunized with JHMV. Lane 1, JHMV-infected cells; lane 2, VVJN-infected cells; lane 3, VVJNs-infected cells; lane 4, VVJNn-infected cells; lane 5, VVJNa-infected cells; lane 6, vSC8-infected cells.

N protein-specific CTL activity

Initially, mice were immunized with either JHMV or VVJN. Spleen cells were prepared from the immunized mice after 3–8 weeks and were cultured with syngeneic irradiated feeder cells infected with either JHMV or VVJN. Figure 3A shows that spleen cells from mice immunized with JHMV and stimulated for 6 days in vitro with JHMV-infected feeder cells specifically lysed J774.1 targets infected with JHMV. Little or no lysis was observed on uninfected cells. In addition, no reactivity was observed using sera from Balb/c or C57Bl/6 mice immunized with JHMV, suggesting the absence of an immunoglobulin epitope(s) in this region.

N-specific CTL activity is restricted to the carboxy terminus

To determine the location of the epitope(s) recognized by the N-specific, Ld-restricted CTL, Balb/c mice were immunized with JHMV and the spleen cells cultured for 6 days with irradiated syngeneic VVJN-infected feeder cells. The Ld-expressing K2A7 cell line was infected with vSC8, VVJN, VVJNs, VVJNn, and VVJNa at an m.o.i. of 50. Figure 5A shows that CTL activity was observed only when targets were infected with VVJN. No lysis over background (vSC8) was observed for targets infected with recombinant vaccinia viruses expressing any of the carboxy-terminal deletions, (VVNs, VVJNn, and VVJNa), indicating that the epitope(s) is contained within the carboxy-terminal 149 amino acids. Sequence analysis of the gene encoding the N protein derived from a number of MHV strains demonstrated a region of heterogeneity within the carboxy terminus (Parker and Masters, 1990). To determine if the epitope(s) was contained within this variable region, the ability of CTLs induced by immunization with JHMV to recognize J774.1 targets infected with the A59 strain of MHV was tested. The data in Fig. 5B shows that the targets infected with the A59 strain of MHV were effectively recognized by these JHMV-in-
MHV-SPECIFIC CYTOTOXIC T LYMPHOCYTE RESPONSE

**Fig. 3.** JHMV-specific CTL activity. J774.1 cells were infected with JHMV, VVJN, or vSC8 and labeled with $^{51}$Cr 6 hr postinfection. The percentage specific $^{51}$Cr release was determined at 4 hr after incubating $1 \times 10^4$ targets with the effectors derived from the secondary cultures at various E:T ratios. (A) Spleen cells from JHMV-immunized mice stimulated in vitro for 6 days with irradiated (2500R) syngeneic spleen cells infected with JHMV (m.o.i. of 0.05). (B) Spleen cells from JHMV-immunized mice stimulated for 6 days with irradiated syngeneic spleen cells infected with VVJN (m.o.i. of 5 to 10). (C) Spleen cells from VVJN-infected mice stimulated in vitro for 6 days with irradiated syngeneic spleen cells infected with VVJN (m.o.i. of 5 to 10). (D) Spleen cells from JHMV-infected mice were stimulated in vitro for 6 days with irradiated syngeneic spleen cells infected with VVJN (m.o.i. of 5 to 10). Prior to addition to the targets the effectors were treated with either anti-CD8 mAb plus complement, anti-CD4 mAb plus complement or with complement only.

duced CTLs, suggesting that the epitope is not within this variable region.

**DISCUSSION**

To begin to understand the repertoire of the JHMV-specific CTL response and the contribution of this population of effectors to acute and chronic demyelinating disease, we have constructed recombinant vaccinia viruses that express the JHMV N protein and a series of deletions from the carboxy terminus. CTLs specific for JHMV have been described previously (Yamaguchi et al., 1988) and recent experiments using these clones have shown that they can protect infected recipients, presumably via the reduction of infectious virus (Yamaguchi et al., 1991). However, neither the restricting element nor the viral protein recognized by these clones has been reported. The N protein plays multiple roles in the structure and replication of coronaviruses, including the encapsidation of genomic RNA to form a helical nucleocapsid structure (Lai, 1990). It is also the most abundant protein in MHV-infected cells, and its corresponding mRNA is the most abundant species detected in infected cells (Spaan et al., 1988; Lai, 1990).
Mabs specific for the N protein protect mice from MHV-induced disease, although the epitope(s) recognized have not been determined (Nakanaga et al., 1986, 1987; Lecomte et al., 1987). In addition, the sequence of the N protein coding region is highly conserved among a number of MHV strains (Parker and Masters, 1990), confirming that it plays a vital role in the viral replication cycle. Sequence comparisons showed only two regions of divergence, neither of which are coincident with the CTL epitope described in this report, suggesting that the CTL response is directed against an evolutionarily conserved epitope.

Secondary in vitro cultures of spleen cells from JHMV-immunized mice were examined for virus-specific CTL activity to avoid the possibility of the B cell-mediated nonspecific lysis of MHV-infected targets ex-
pressing the S protein (Wysocka et al., 1989). CTL activity was detected when JHMV-infected targets were tested, confirming that CTLs can be induced in Balb/c mice and indicating that analysis of these cultures would help determine the viral proteins recognized. Although clonal populations of anti-JHMV CTL have been reported previously (Yamaguchi et al., 1988), the 31-kb coding capacity of the MHV genome has hindered a determination of the proteins recognized by these clones. The JHMV N protein-specific CTLs demonstrated in this report were examined for their restriction elements using L929 (H-2k) cells transfected with the three Class 1 genes derived from the H-2d haplotype. Analysis of the ability of the anti-N protein-specific CTL to recognize these three cell lines clearly showed that the response was restricted to the Ld molecule. Reombinant vaccinia viruses expressing the carboxy-terminal deletions of the N protein were used to localize the domain(s) of the N protein recognized in the context of the Ld molecule. This analysis demonstrated that only recombinant vaccinia virus expressing the entire N protein was recognized. No CTL activity was detected with recombinant vaccinia virus expressing the N protein from which the carboxy-terminal 149 amino acids had been deleted nor any of the recombinant vaccinia viruses expressing additional carboxy-terminal deletions. These data suggest the epitope(s) of the JHMV N protein recognized is contained within the carboxy-terminal portion of the N protein. The precise epitope has not been defined nor can our present data rule out the presence of additional epitopes at the sites of truncations. It is likely, however, by analogy with other viral-specific CTL responses (Bastin et al., 1987; Whitton et al., 1988), that only one or at most a few epitopes are contained within the domain comprising amino acids 307 to 455. A hypervariable domain (amino acids 381–405) has been found within this region (Parker and Masters, 1990); however, our data demonstrate that JHMV-specific CTL can recognize J774.1 targets infected with either MHV-A59 (Fig. 5) or MHV-S (data not shown), each of which differ from JHMV in this region. These data suggest that the epitope recognized by the JHMV-specific CTLs is not contained within this hypervariable region.

Immunization with either JHMV or VV/JN protected mice from a lethal JHMV infection (unpublished); however, both groups of mice also produced anti-N antibodies. Thus, the direct role of N-specific CTL activity in protection is not yet clear. Preliminary data also indicate that mAb specific for the N protein can provide protection from JHMV; however, similar to the other models of mAb-mediated protection, there is no reduction of virus within the CNS (unpublished). Therefore, the possibility that antibody epitopes contained within the carboxy terminus are able to confer protection cannot be ruled out. The isolation and characterization of clonal populations of anti-N-specific CTLs are currently in progress in order to precisely map the epitope and to examine the role of these cells in JHMV-induced CNS disease. Comparison of the epitope(s) within the carboxy terminus recognized by antibody and CTLs should allow a clear distinction to be made between the roles of the anti-N antibodies and the CTL activity in both the acute and chronic forms of JHMV-induced demyelination in mice. It will also be of interest to determine the number of mouse haplotypes capable of recognizing this epitope, since preliminary experiments indicate that JHMV is not able to induce an N protein-specific CTL response in C57BL/6 mice (data not shown: Perlman, personal communication).

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