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Immunogenic Determinants of a Neuropathogenic Murine Leukemia Virus

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Previous studies of Cas-Br-M murine leukemia virus (MuLV) (Cas-MuLV) infection demonstrated that cytotoxic T cells (CTL) of the CD8$^+$ phenotype play a role in resistance to the neuropathogenic effects of the virus in NFS/N mice. In the current study, we sought to identify the Cas-MuLV epitopes that are immunogenic for the CTL response. Infection of adult NFS/N mice with a well-characterized neuropathogenic variant of Friend MuLV, PVC-211 MuLV (PVC-MuLV), was not immunogenic for MuLV-specific CTL. Therefore, we constructed chimeric viruses between Cas-MuLV and PVC-MuLV. Infectious chimeras contained the Cas-MuLV *env* gene on a PVC-MuLV background (PVC-Cas^{env}MuLV) and the PVC-MuLV *env* gene on a Cas-MuLV background (Cas-PVC^{env}MuLV). Cas-MuLV-specific CTL were found following inoculation of both the chimeric viruses and the parental Cas-MuLV but not the parental PVC-MuLV, despite evidence of antibody responses to both parental and chimeric MuLV. CTL generated in response to infection with PVC-Cas^{env}MuLV and Cas-PVC^{env}MuLV were exclusively of the CD8$^+$ phenotype. These results indicate that both the *env* and *gag-pol* regions of Cas-MuLV express epitopes that are immunogenic for CTL.

Previous studies of Cas-Br-M murine leukemia virus (MuLV) (Cas-MuLV) infection have demonstrated that immune T cells play a role in resistance to the neuropathogenic effects of the virus (4, 6, 20). CD8$^+$ cytotoxic T cells (CTL) derived from syngeneic mice exposed to Cas-MuLV at 21 days of age conferred resistance to the neuropathogenic effects of Cas-MuLV when transferred into susceptible newborn NFS/N mice (4). However, since protective T-cell immune responses have not been universally demonstrated in mice during the course of other neuropathogenic MuLV infections (5), the Cas-MuLV epitopes immunogenic for CTL induction in adult NFS/N mice may be unique. Since the *env* sequences have been implicated in the neuropathogenic effects of several MuLV strains (2, 12, 16, 25) and are the least conserved among the MuLV family of retroviruses (17, 20), we focused our attention on the potential immunogenic epitopes within the Cas-MuLV *env* gene.

In this study, chimeric viruses were constructed between Cas-MuLV (10) and PVC 211 MuLV (PVC-MuLV) (12), a neuropathogenic variant of Friend MuLV that did not stimulate the induction of retrovirus-specific CTL. The results of this study demonstrated that Cas-MuLV contains epitopes in the *env* region and also in the *gag-pol* region that are immunogenic for CTL in adult NFS/N mice.

MATERIALS AND METHODS

Mice and virus stocks. Pregnant NFS/N NCr mice were obtained from the Animal Genetics and Production Branch, National Cancer Institute, Frederick, Md., or from the NFS/N breeding colony maintained at the small-animal facility of the Veterans Affairs Medical Center, Baltimore, Md.

The pBR-NE-8 molecular clone of Cas-MuLV was generously provided to us by P. Jolicoeur (10). The full-length molecular clone of PVC-MuLV, 3d-1L MuLV, was previously described (12) and was provided to us by M. Masuda. Stocks of Cas-MuLV, PVC-MuLV, and infectious chimeras containing the Cas-MuLV *env* gene on a PVC-MuLV background (PVC-Cas^{env}MuLV) and the PVC-MuLV *env* gene on a Cas-MuLV background (Cas-PVC^{env}MuLV) were prepared by calcium phosphate transfection of ligated DNA into NIH 3T3 cells (26). Multiple stocks of parental and chimeric MuLV were produced following independent transfections of full-length DNA into NIH 3T3 cells. Virus-containing supernatants were assayed by X-C plaque assay (23) to determine titers of infectious virus.

NFS/N mice were inoculated intraperitoneally at 2 or 21 days of age with 0.03 or 0.05 ml, respectively, of Cas-MuLV, PVC-MuLV, PVC-Cas^{env}MuLV, or Cas-PVC^{env}MuLV containing approximately 1×10^3 to 5×10^3 PFU. Multiple stocks of MuLV were used for these studies, and all were neuropathogenic. Control mice for virus-infected NFS/N mice were inoculated with equivalent volumes of media.

Construction of chimeric MuLV. The parental retroviruses used in the construction of PVC-Cas^{env}MuLV were PVC-211-3d-IL, cloned into the *Eco*RI site of pUC19, and the Cas-Br-E molecular clone pBR-NE-8, also cloned into the *Sal*I site of pUC19 (Fig. 1). For construction of chimeric genomes between Cas-MuLV and PVC-MuLV, the *Pvu*I and *Sph*I sites of the pUC19 vector were deleted by partial digestion with *Pvu*I and complete digestion with *Sph*I. The overhangs generated by these enzymes were filled in with the Klenow fragment of DNA polymerase ligated together with T4 DNA ligase. Deletion of these sites was confirmed by the inability of *Pvu*I and *Sph*I enzymes to linearize the newly constructed pUC19 deletion (pUC-D) vector. Complete Cas-MuLV and PVC-MuLV fragments were subcloned into the pUC-D vector. DNA fragments used in the constructions were purified from agarose gels with a Qiaex gel extraction kit (Qiagen, Chatsworth, Calif.), and DNA-modifying enzymes were from Bethesda Research Laboratories (Gaithersburg, Md.).

PVC-Cas^{env}MuLV was constructed by digesting the pUC-D-PVC plasmid with *Cla*I and *Sph*I to remove the 2.5-kb 3′ *pol-env* sequences. The pUC-D-NE8 plasmid was first digested to completion with *Cla*I and then partially digested with *Sph*I. The resulting 2.5-kb Cas-MuLV 3′ *pol-env* fragment was separated by gel electrophoresis and purified as described above. This fragment was ligated into the gel-purified 8.4-kb *Sph*I-*Cla*I fragment of pUC-D-PVC to generate a chimeric plasmid.

The reciprocal Cas-PVC^{env}MuLV was generated by using the same restriction sites as were used to generate PVC-Cas^{env}MuLV. In this instance, the 8.4-kb *Sph*I-*Cla*I fragment was derived from the pUC-D-NE8 plasmid and the 2.5-kb 3′ *pol-env* fragment was generated by digestion of the pUC-D-PVC plasmid.

The pUC-D-NE8 clone has a unique *Sal*I site and two *Sph*I sites, one of which is within the *env* gene. The pUC-D-PVC clone has only one *Sph*I site and a unique *Eco*RI site. To determine that the correct *env* region was cloned into the appropriate parent, the chimeric constructs were digested with the unique parental restriction enzyme and *Sph*I.

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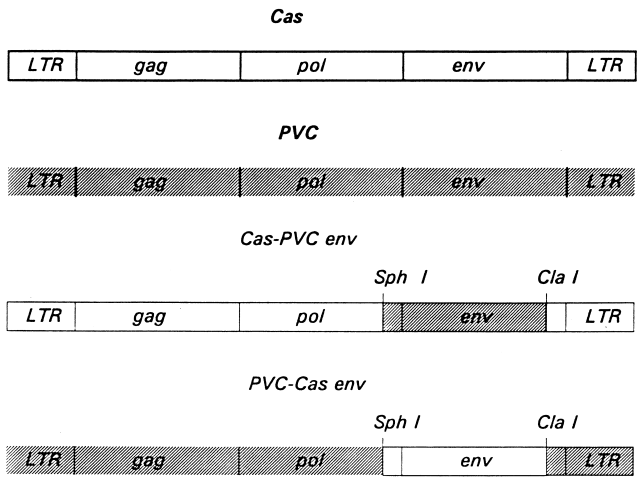

FIG. 1. Schematic diagrams of the unpermuted parental and chimeric MuLV. The restriction enzyme sites used in the construction of the chimeras do not correspond exactly to the boundaries of the viral genes. Both of the chimeric MuLV strains generated reverse transcriptase activity after transfection into NIH 3T3 cells and were infectious.

Following transformation into DH5α competent cells (Gibco-BRL, Gaithersburg, Md.), the chimeric plasmids were transfected into NIH 3T3 cells by the calcium phosphate transfection system (Gibco-BRL) and the culture supernatants were screened for reverse transcriptase activity (3).

MuLV-specific cytotoxicity assay. Cytotoxicity directed against Cas-MuLV- or PVC-MuLV-infected targets was measured 4 to 12 weeks postinfection by secondary in vitro stimulation of spleen cells from adult NFS/N mice inoculated with parental MuLV, chimeric MuLV, or media. Single-cell suspensions of splenocytes from MuLV-infected mice were cocultured for 5 days at 37°C in the presence of gamma-irradiated splenocytes derived from NFS/N mice neonatally infected with the corresponding MuLV. In some experiments, the B-cell lymphoma cell line NS467, derived from a Cas-MuLV-infected NFS/N mouse (15), was also used for in vitro stimulation as previously described (21). Briefly, NS467 cells or lipopolysaccharide (LPS)-stimulated splenocytes from mice neonatally infected with Cas-MuLV or PVC-MuLV were used as target cells following labeling with ^{51}Cr. Various numbers of responder cells were added to 3×10^4 target cells, producing killer-to-target cell ratios ranging from 40:1 to 5:1. After a 4-h incubation period at 37°C, ^{51}Cr release was measured from 100 μl of culture supernatant from the wells containing killer cells and labeled targets (experimental release), target cells alone (spontaneous release), and labeled target cells lysed in water (total release). The percentage of cytotoxicity was determined by the following formula: [^{51}Cr release (experimental) − ^{51}Cr release (spontaneous)]/[^{51}Cr release (total) − ^{51}Cr release (spontaneous)]. Results were expressed as mean percent cytotoxicity ± standard deviation.

Cellular ELISA. Antibody directed against MuLV proteins was detected with a cellular enzyme-linked immunosorbent assay (ELISA) developed in our laboratory. NIH 3T3 fibroblast cells (American Type Culture Collection, Rockville, Md.) were transfected with DNA from Cas-MuLV and used as the source of viral antigen in the ELISA. Greater than 95% of the transfected NIH 3T3 cells expressed MuLV gp70, as detected by indirect immunofluorescence performed with a polyclonal goat anti-Rauscher gp70 antibody (data not shown). Flat-bottom 96-well tissue culture plates were precoated with 0.2% porcine skin gelatin (type A, 60 Bloom; Sigma, St. Louis, Mo.) for 3 h at 4°C and washed two times with phosphate-buffered saline (PBS) prior to use.

Transfected NIH 3T3 cells in Dulbecco's medium (high glucose) containing 10% fetal bovine serum and penicillin-streptomycin (Gibco, Grand Island, N.Y.) were plated into wells of precoated plates at a concentration of 10^4 per well and allowed to grow to confluence (between 18 and 24 h of incubation at 37°C). Plates were washed three times in PBS, and 100 μl of freshly prepared Dulbecco's PBS containing 1% bovine serum albumin (BSA) was added to each well. Following a 1-h incubation at room temperature, serial twofold dilutions of mouse serum in PBS–1% BSA starting at a dilution of 1/10 were added to wells in triplicate. As a control, PBS–1% BSA without mouse serum was added to wells. After a 2-h incubation at room temperature, the plate was washed four times with PBS and an alkaline phosphatase-conjugated secondary antibody (goat anti-mouse immunoglobulin G, heavy and light chains; Jackson Immunoresearch Laboratories, West Grove, Pa.) was added at a 1/200 dilution. Following a 1-h incubation at room temperature and four washes with PBS, freshly prepared enzyme substrate (*p*-nitrophenyl phosphate; Sigma catalog number 104) at 1 mg/ml in diethanolamine buffer was added to each well, and the plate was incubated at 37% for 15 min to allow for reaction product development. Optical density was measured at 405 nm on a Molecular Devices plate reader. Sera from naive mice were included in all assays, and test sera were considered positive at a given dilution if the optical density at 405 nm was at least twofold greater than the optical density reading of control sera at the same dilution. For the detection of anti-PVC-MuLV antibody, NIH 3T3 cells transfected with PVC-MuLV were used as the source of antigen. Similar titers of anti-PVC MuLV were detected with this assay (data not shown).

T-cell fractionation into CD4- and CD8-enriched cell populations. Splenocytes from MuLV-inoculated NFS/N mice, cocultured for 5 days with gamma-irradiated NS467 cells, were enriched for $CD4^+$ and $CD8^+$ T cells by high-affinity negative selection using commercially available columns as described by the manufacturer (R&D Systems, Minneapolis, Minn.). The purity of each T-cell population was determined by flow cytometry using fluorescence-labeled anti-Thy 1.2, anti-CD4, and anti-CD8 monoclonal antibodies (Becton Dickinson, Mountain View, Calif.) with an Epics Elite flow cytometer (Coulter, Hialeah, Fla.). The purity of CD4- and CD8-enriched populations was routinely ≥95%.

RESULTS

CTL response following adult inoculation of NFS/N mice with parental or chimeric MuLV. NFS/N mice inoculated with 1×10^3 to 5×10^3 PFU of molecularly cloned Cas-MuLV at 21 days of age developed CTL directed at Cas-MuLV-infected cells. In contrast, 21-day-old mice inoculated with an equivalent dose of PVC-MuLV failed to develop a PVC-MuLV-specific CTL response (Fig. 2). CTL derived from Cas-MuLV-infected NFS/N mice failed to kill PVC-MuLV-infected target cells, demonstrating the specificity of the response to Cas-MuLV (Fig. 2). Allogeneic responder cells from CBA/JHsd mice were able to lyse PVC-MuLV-infected, LPS-stimulated splenocytes (75% specific cytotoxicity at a killer-to-target cell ratio of 10:1), demonstrating that these target cells were in fact sensitive to CTL-mediated cytotoxicity.

Cas-MuLV-specific CTL were also induced following inoculation of 21-day-old NFS/N mice with equivalent doses of PVC-Cas^{env}MuLV or Cas-PVC^{env}MuLV (Fig. 3). Both PVC-Cas^{env}MuLV and Cas-PVC^{env}MuLV induced CTL responses

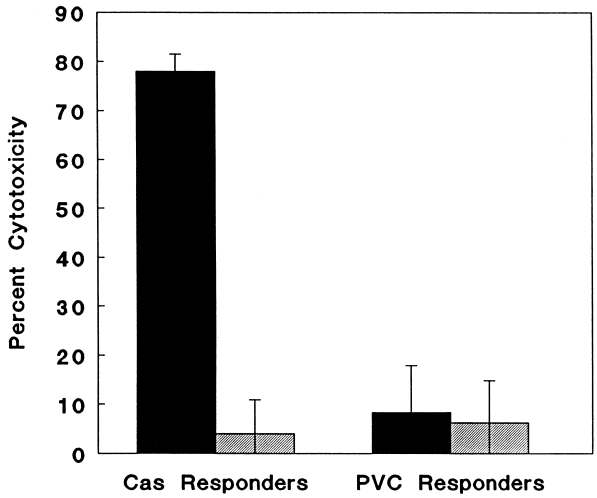

FIG. 2. Specificity of CTL response following inoculation with parental MuLV. Twenty-one-day-old NFS/N mice were inoculated intraperitoneally with 1×10^3 to 5×10^3 PFU of molecularly cloned Cas-MuLV or PVC-MuLV. Splenocytes were isolated from Cas-MuLV-infected NFS/N mice 8 to 12 weeks postinfection and restimulated in vitro with splenocytes derived from mice neonatally infected with Cas-MuLV and examined in a 4-h ^{51}Cr release assay for CTL activity directed at Cas-MuLV-infected splenic blast target cells (n = 3) (■) or PVC-MuLV-infected blast cells (n = 4) (▨) (killer/target cell ratio = 20). Similarly, splenocytes from PVC-MuLV-infected NFS/N mice were restimulated with splenocytes from NFS/N mice neonatally infected with PVC-MuLV. Cytotoxicity was measured with Cas-MuLV-infected target cells (n = 4) or PVC-MuLV-infected target cells (n = 5). Results are expressed as mean percent cytotoxicity ± standard deviation.

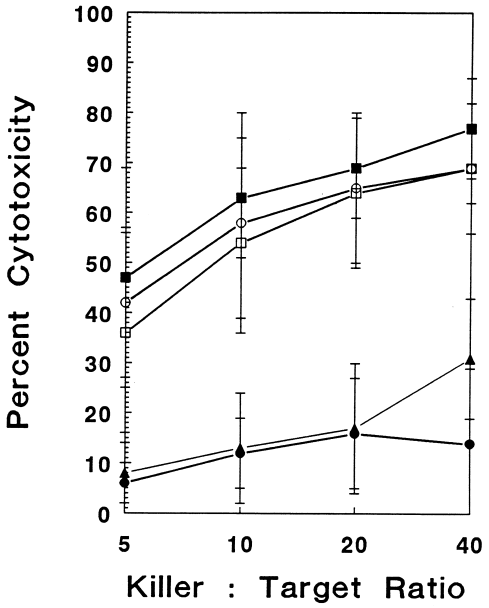

FIG. 3. CTL response following inoculation with chimeric MuLV. NFS/N mice (for PVC-MuLV, $n = 7$; for other viruses, $n = 4$) were inoculated at 21 days of age with 1×10^3 to 5×10^3 PFU of Cas-MuLV (○), PVC-MuLV (●), PVC-Cas^{env}MuLV (□), Cas-PVC^{env}MuLV (■), or media (▲). Between 4 and 12 weeks postinfection, splenocytes from these NFS/N mice were restimulated in vitro with NS467 cells. Cytotoxicity against ^{51}Cr-labeled NS467 cells was measured in a 4-h ^{51}Cr release assay. Results are expressed as mean percent cytotoxicity ± standard deviation.

against Cas-MuLV target cells comparable to those induced by the parental Cas-MuLV at all killer-to-target ratios tested (within 1 standard deviation). In contrast, responding splenocytes from PVC-MuLV-infected NFS/N mice demonstrated levels of cytotoxicity comparable to or less than background levels of killing observed in control splenocytes (Fig. 3). Effector cells from mice inoculated with either chimeric virus failed to lyse PVC-MuLV-infected target cells (data not shown).

Antibody production in MuLV-infected NFS/N mice. To ensure that the lack of a CTL response to PVC-MuLV was not due to a failure of the virus to induce an immune response in adult NFS/N mice, antibody to MuLV was measured in a cellular ELISA. There was a readily detectable anti-MuLV antibody response to PVC-MuLV, Cas-MuLV, PVC-Cas^{env} MuLV, and Cas-PVC^{env}MuLV in mice infected as adults (Fig. 4). In contrast, uninfected mice failed to generate a measurable anti-MuLV antibody response in this assay system.

Phenotypic analysis of CTL induced by chimeric MuLV. In previous studies, CTL generated in response to a biological clone of Cas-MuLV were demonstrated to be exclusively CD8$^+$ (20) and were highly protective in vivo (4). In this study, using negative selection, CD4- and CD8-enriched T-cell populations were isolated from splenocytes of adult NFS/N mice inoculated with PVC-Cas^{env}MuLV and Cas-PVC^{env}MuLV following in vitro restimulation with NS467 cells. High levels of MuLV-specific cytotoxicity were found in CD8-enriched populations at all killer-to-target cell ratios tested (Fig. 5). In contrast, CD4-enriched populations from PVC-Cas^{env}MuLV- and Cas-PVC^{env}MuLV-infected splenocytes had negligible levels of cytotoxicity directed against MuLV-expressing target cells.

DISCUSSION

In this study, it was demonstrated that an infectious molecular clone of Cas-MuLV (pNE8) induced an MuLV-specific CTL response when inoculated into adult NFS/N mice (Fig. 2 and 3). The MuLV-specific response to the infectious molecular clone was similar to that previously shown to a biological clone of Cas-MuLV known as Cas-Br-M MuLV (21). In contrast, a molecular clone of PVC-MuLV (3d-IL) (12) did not stimulate an MuLV-specific CTL response in adult NFS/N mice (Fig. 2 and 3).

The failure to induce CTL was not due to an inability of PVC-MuLV to generate an immune response, as MuLV-specific antibody was produced (Fig. 4). While we did not specifically examine the mechanisms responsible for the absence of a PVC-MuLV-specific CTL response, FrCas^{E}MuLV, a chimeric virus generated from Friend MuLV and 15-1 MuLV (19), generated CTL in adult NFS/N mice, suggesting that the presence of a Friend MuLV background did not result in general immunosuppression (data not shown).

The CTL generated in response to Cas-MuLV were not cytotoxic to PVC-MuLV-infected spleen cells (Fig. 2). This specificity of the MuLV CTL response further suggested that the CTL were not recognizing cross-reactive sequences in highly conserved regions of the two neuropathogenic MuLV strains. These results also suggested that immunogenic differences exist between the neuropathogenic MuLV and that these differences could be used to identify those sequences in Cas-MuLV that are immunogenic for a CTL response.

To test this hypothesis, chimeric MuLVs (PVC-Cas^{env}MuLV and Cas-PVC^{env}MuLV) were constructed between the two parental neuropathogenic MuLV strains, one of which was immunostimulatory for CTL (Cas-MuLV) and one which was not (PVC-MuLV).

The detection of Cas-MuLV-specific CTL following inoculation of PVC-Cas^{env}MuLV (Fig. 3) identified the presence of

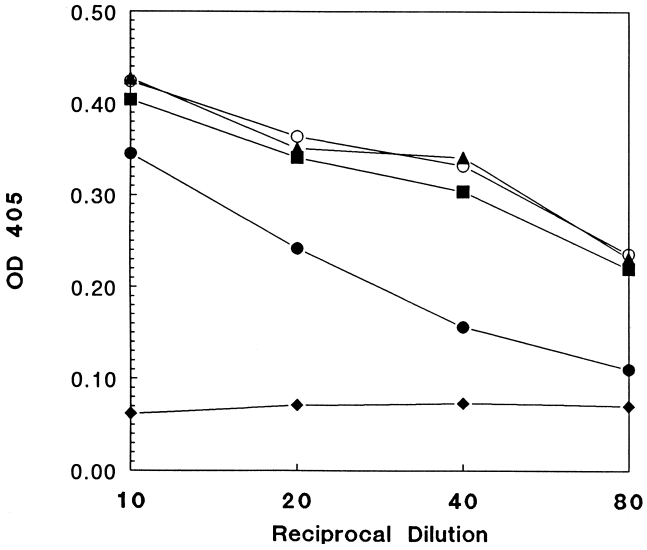

FIG. 4. Antibody response to parental and chimeric MuLV. By a cellular ELISA, an anti-MuLV antibody response was measured in NFS/N mice inoculated at 21 days of age with media (◆), Cas-MuLV (○), PVC-MuLV (■), PVC-Cas^{env}MuLV (●), or Cas-PVC^{env}MuLV (▲). Sera were collected between 4 and 12 weeks postinfection. In this representative experiment, twofold dilutions from an initial 1:10 dilution were applied to wells containing NIH 3T3 cells infected with Cas-MuLV. Results are expressed as optical density readings at 405 nm (OD 405).

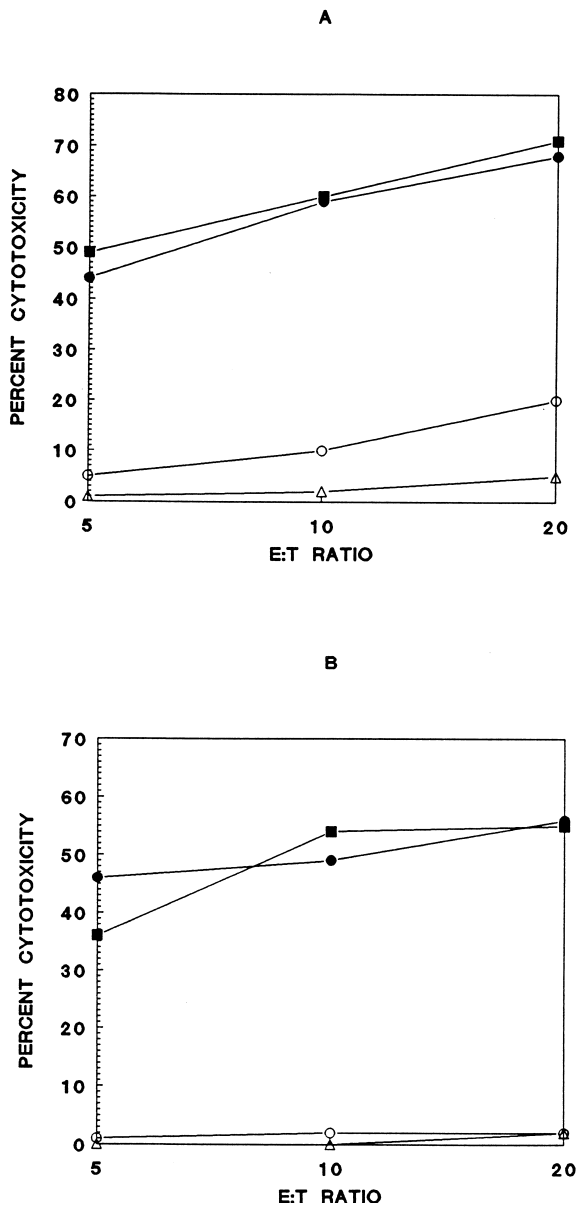

FIG. 5. Phenotype of CTL in response to chimeric MuLV. Four to twelve weeks postinfection, splenocytes were isolated from NFS/N mice inoculated with PVC-Cas^{env}MuLV (A) or Cas-PVC^{env}MuLV (B) at 21 days of age. As controls, splenocytes were isolated from NFS/N inoculated with media at 21 days of age. Following an in vitro restimulation with NS467 cells, cytotoxicity directed against ^{51}Cr-labeled NS467 cells by unfractionated control splenocytes (△), unfractionated splenocytes (●), CD4$^+$-enriched cells (○), or CD8$^+$-enriched T cells (■) was measured in a 4-h ^{51}Cr release assay. Results are expressed as mean percent cytotoxicity and represent data from the pooled splenocytes of two to three NFS/N mice in three independent experiments. E:T, effector-to-target.

viral epitopes immunogenic for CTL induction in the *env* gene of Cas-MuLV. This was not too surprising, since the amino acid sequences of Cas-MuLV and PVC-MuLV share only 79.2% homology (17, 20). However, Cas-PVC^{env}MuLV inoculation also resulted in the generation of CTL epitopes outside of the *env* gene (Fig. 3). There is 92 to 96% amino acid homology between the *gag-pol* regions of Cas-MuLV and PVC-MuLV (17, 20), which suggests that the immunogenic epitope(s) for Cas-MuLV-specific CTL in this region can be found in the 4 to 8% of the disparate amino acid sequences.

Previous studies identified Cas-MuLV-specific CTL as CD8$^+$ T cells (4, 21). Similarly, in this study both of the chimeric constructs generated a CTL response that was mediated by CD8$^+$ T cells (Fig. 5).

CTL responses have been described during infection with a number of MuLV strains, including Friend (7, 9, 13, 22), Gross (18), and Rauscher (8). However, the role of retrovirus-specific CTL in neurologic disease pathogenesis has been described only for Cas-MuLV (4, 21) and *ts*1 Moloney MuLV (*ts*1-MuLV) (24) infections. In Cas-MuLV infections, CD8$^+$ CTL transferred to syngeneic neonatal NFS/N mice protects them from neurologic disease induction following Cas-MuLV challenge while unprotected neonatal NFS/N mice develop neurologic disease from 3 to 5 weeks following challenge (21). Since Cas-MuLV still replicates in the brains and spleens of CTL recipients without evidence of clinical disease (reference 4 and unpublished data), the CTL may function by preventing virus dissemination to the central nervous system (CNS) during the critical period for susceptibility. PVC-MuLV fails to elicit CTL following infection of either adult or neonatal NFS/N mice. The period of vulnerability to CNS disease is even more restricted (4 days) than that following Cas-MuLV infection ($\leq$10 days). Therefore, it is unlikely that CTL play a role in the prevention of retrovirus dissemination to the CNS and in the alteration of disease expression following PVC-MuLV infection. Ongoing syngeneic transfer studies in our laboratory are examining the possibility of different biological effects of CTL resulting from PVC-Cas^{env}MuLV or Cas-PVC^{env}MuLV infection.

The CTL response to both *ts*1-MuLV and Cas-MuLV is mediated by CD8$^+$ T-cell populations (21, 24). However, protection against *ts*1-MuLV-induced neurologic disease was enhanced by the presence of CD4$^+$ cells (24). In previous studies, we showed that CNS protection against Cas-MuLV infection was not enhanced by the presence of CD4$^+$ T cells (21). These differing results suggest that the immune mechanisms in response to these two neuropathogenic MuLV strains are not the same. This might be expected since CNS disease induced by Cas-MuLV infection as opposed to *ts*1-MuLV infection occurs at a later time (21, 24).

The demonstration of CTL directed against both *env* and *gag-pol* gene products is not unique to neuropathogenic MuLV. Similar CTL have been described in other MuLV models of tumorigenesis and immunity (7, 11, 22). Spontaneous recovery from Friend MuLV-induced leukemia is mediated by *env*-specific CD8$^+$ T cells, with CD4$^+$ T cells implicated in the maintenance of the recovery (22). In another study, CD4$^+$ T-helper cells, induced following immunization with a recombinant vaccinia virus expressing the Friend MuLV *gag* gene, protectively immunized against Friend MuLV-induced leukemia (14).

Recent studies of human immunodeficiency virus infection demonstrated that CD8$^+$ CTL activity may play an important role in the control of viremia (1). Cas-MuLV-specific CTL also appear to work at the level of virus load reduction (20a) by clearing retrovirus-infected cells and delaying a critical interaction between the retrovirus and the CNS (4). Further studies of epitope-specific CTL induction in MuLV infection may be useful as a model for CTL induction in vaccine development strategies for human retroviral diseases.

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