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Mouse Hepatitis Virus-Specific Cytotoxic T Lymphocytes Protect from Lethal Infection without Eliminating Virus from the Central Nervous System

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Acute infection of the central nervous system by the neurotropic JHM strain of mouse hepatitis virus (JHMV) induces nucleocapsid protein specific cytotoxic T lymphocytes (CTL) not found in the periphery (S. Stohlman, S. Kyuwa, J. Polo, D. Brady, M. Lai, and C. Bergmann, J. Virol. 67:7050–7059, 1993). Peripheral induction of CTL specific for the nucleocapsid protein of JHMV by vaccination with recombinant vaccinia viruses was unable to provide significant protection to a subsequent lethal virus challenge. By contrast, the transfer of nucleoprotein-specific CTL protected mice from a subsequent lethal challenge by reducing virus replication within the central nervous system, demonstrating the importance of the CTL response to this epitope in JHMV infection. Transfer of these CTL directly into the central nervous system was at least 10-fold more effective than peripheral transfer. Histological analysis indicated that the CTL reduced virus replication in ependymal cells, astrocytes, and microglia. Although the CTL were relatively ineffective at reducing virus replication in oligodendroglia, survivors showed minimal evidence of virus persistence within the central nervous system and no evidence of chronic ongoing demyelination.

Infection with the neurotropic JHM strain of mouse hepatitis virus (JHMV) produces an acute encephalitis with primary demyelination (27, 56). Survivors of the acute infection with either the wild type or variants of JHMV are unable to completely clear virus from the central nervous system (CNS) and exhibit histological evidence of chronic ongoing CNS demyelination (14, 16, 26). However, little or no infectious virus can be isolated from these mice (51). The similarities of chronic JHMV infection, i.e., focal areas of primary demyelination, coupled with the inability to recover infectious virus from the majority of infected mice, suggest parallels to the human demyelinating disease multiple sclerosis. Indeed, JHMV infection produces an acute demyelinating encephalitis in primates, and coronaviruses have been implicated as potential etiological agents of multiple sclerosis (22, 31, 32, 42).

During acute JHMV infection, the host's antiviral immune response not only determines the clinical outcome but also appears to play a critical role in establishing the chronic form of infection (16, 26). For example, immunocompromised hosts are unable to clear virus and succumb to a lethal panencephalitis (26, 41, 54, 56). By contrast, protection can be achieved via manipulations of the antiviral immune response (26). In contrast to virus infections in which a single major effector mechanism predominates, JHMV infection can be modulated by cell-mediated immunity as well as both neutralizing and nonneutralizing antibodies (10, 17, 29, 33). During infection, all types of immune effectors are recruited into the CNS; however, $CD8^+$ T cells appear to be locally expanded or recruited more rapidly than other cell types (12, 48, 59). In addition to antiviral antibodies, both virus-specific $CD4^+$ Th1 T cells and $CD8^+$ cytotoxic T lymphocytes (CTL) provide protection from acute disease (26, 49, 52, 55, 62, 63). In general, neither the nonneutralizing antibodies nor the $CD4^+$ T cells are effective in reducing virus replication within the CNS (26). Although some $CD4^+$ T-cell clones have been reported to reduce JHMV titer within the CNS (23, 62), this may be a consequence of help provided to CTL (18, 52). Therefore, protection mediated by these forms of immunotherapy which do not clear infectious virus results in increased virus-induced immunopathology (26, 62, 63). These data suggest that a broad host immune response to JHMV infection which provides protection but is unable to completely clear virus from the CNS.

A primary role for CTL in protection from JHMV has been suggested by a number of studies; however, it appears that the induction of these cells requires $CD4^+$ T-cell-mediated help (18, 52, 58). At least two populations of JHMV-specific $CD8^+$ CTL are induced in BALB/c mice, one restricted to an epitope within a nonstructural protein(s) which may be restricted to major histocompatibility complex (MHC) class I K^d (62) and a second MHC class I L^d-restricted CTL population specific for an epitope comprising amino acids 318 to 326 (APTAGAFFF) of the structural nucleocapsid (N) protein (5, 47). The CTL response within the CNS of JHMV-infected mice is composed predominantly of CTL restricted to the N protein, although CTL specific for a nonstructural protein(s) as well as $CD4^+$ cells with cytolytic activity are also present (48). By contrast, examination of the spleen and cervical lymph nodes showed that these compartments contained only CTL specific for nonstructural protein(s) (48). These data suggested that the CTL response specific for the N protein may be critical in the pathogenesis and clearance of JHMV from the CNS.

In this paper, we report that the adoptive transfer of CTL specific for an L^d-restricted epitope within the N protein of JHMV can protect BALB/c ($H\text{-}2^d$) mice and mice congenic at L^d from a lethal challenge. Protection results from a reduction of virus within the CNS; however, CTL are unable to completely clear virus. Histological analysis shows that following CTL transfer, virus replication is reduced in astrocytes, microg-

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lia, endothelial cells, and perivascular macrophages, while there is little reduction of replication in oligodendroglia. Consistent with an overall reduction of CNS virus replication, little evidence of chronic demyelination or persistent virus was detected following CTL-mediated protection. These data provide a rationale for the multiple components of the immune response required for protection and suppression of virus replication within the CNS during JHMV-induced demyelination.

MATERIALS AND METHODS

Viruses and cell lines. The DM strain, a plaque-purified isolate derived from a suckling mouse brain pool which has the plaque morphology and pathogenesis consistent with parental JHMV (46, 56), was propagated and plaque assayed by using the murine DBT astrocytoma cell line as previously described (46). Virus titers were determined by homogenization of one half of the brain in 2.0 ml of Dulbecco's phosphate-buffered saline, pH 7.4 (PBS), using a Ten Broeck tissue homogenizer. The remainder was taken for histopathology (see below). Following centrifugation at 1,500 × *g* for 20 min at 4°C, supernatants were assayed immediately or frozen at −70°C. Virus titers were determined by plaque assay, using monolayers of DBT cells as previously described (46). Data presented are the average titers of groups of three or more mice. The construction, purification, and characterization of the recombinant vaccinia viruses (rVV) expressing the entire 455-amino-acid JHMV N protein (designated vJN455), amino acids 1 to 374 (designated vJN374), amino acids 301 to 351 (vJN51), and amino acids 318 to 326 (designated vJN9) have been previously described (5, 47). The rVV expressing the human immunodeficiency virus strain IIIB gp160 (vPE16) and *Escherichia coli lacZ* gene (vSC8) were kindly provided by B. Moss (National Institutes of Health).

Mice. BALB/c By (*H-2*^{d}), B10.D2 (*H-2*^{d}), C57BL/10 (*H-2*^{b}), and the congenic B10.A(5R) (K^b, D^d, L^d) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) and housed in isolator cages. Donor mice were immunized as previously described (47), using 10^6 PFU of JHMV or 2×10^7 PFU of rVV injected intraperitoneally (i.p.). Mice were infected intracranially (i.c.) with 100 PFU of JHMV contained in 30 μl of PBS. This inoculum is uniformly fatal within 7 to 9 days post infection (p.i.).

Induction and transfer of bulk effector CTL. Spleen cell suspensions were prepared from mice immunized 3 to 8 weeks earlier by i.p. injection of either 2×10^6 PFU of JHMV or 2×10^7 PFU of rVV. Spleen cells (10^8) from immune donors were cultured for 6 days at 37°C in 40 ml of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Gemini Bioproducts), 2 mM glutamine, 25 μg of gentamicin per ml, 1 mM sodium pyruvate, 5×10^{-5} M β-2-mercaptoethanol, and nonessential amino acids (RPMI complete) plus 10% rat concanavalin A supernatant containing 25 mM α-methyl mannopyranoside as previously described (5–7). Spleen cells from mice immunized with JHMV, vJN51, or vJN9 were stimulated by the addition of 1 μM pN318-335 peptide (5). This 18-mer peptide was used due to enhanced solubility compared with the optimal 9-mer. Spleen cells from mice immunized with vPE16 were cultured under identical conditions, using 1 μM p18 peptide as previously described (6, 7). Viable cells were purified by centrifugation using Lympholyte M prior to transfer to naive recipients. Recipients were challenged with JHMV within 6 h of adoptive transfer.

Cytotoxicity assay. Effectors, in 100 μl of RPMI complete supplemented with 5% FCS, were added at various ratios to round-bottom 96-well plates (Falcon Plastics, Oxnard, Calif.). The J774.1 *H-2*^{d} target cell line, obtained from the American Type Culture Collection, was propagated in Dulbecco's modified minimal essential medium containing 10% FCS as previous described (47, 49). J774.1 cells were infected with rVV (multiplicity of infection of 5), incubated overnight at 37°C, and washed in RPMI, 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 three times with RPMI, and resuspended in RPMI containing 5% FCS. Target cells, at a concentration of 10^4 in a 100-μl volume, were added to effector cells. In some experiments, target cells were preincubated for 30 min at 37°C with peptide (pN318-335) containing the immunodominant L^d-restricted N-protein epitope (5, 6). Antibody-plus-complement-mediated depletions were carried out as previously described (47). 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)]/[total (detergent release) − (spontaneous release)]. Maximum spontaneous release values were <20% of total release values.

Histology. For histopathological analysis, mice were sacrificed by CO_2 asphyxiation. Brains and spinal cords were removed. Brains were bisected in the midcoronal plane. Both were fixed for 3 h in Clark's solution (75% ethanol, 25% glacial acetic acid) and embedded in paraffin. Sections were stained with either hematoxylin and eosin or luxol fast blue for routine examination. The distribution of JHMV antigen was examined by immunoperoxidase staining (Vectastain-ABC kit; Vector Laboratories, Burlingame, Calif.) and anti-JHMV monoclonal antibody (MAb) J.3.3 specific for the carboxy terminus of the N protein (44, 54). For identification of cell types staining with JHMV antigen, double immunoperoxidase staining was performed on representative snap-frozen brains. Frozen sections were cut at 6 μm and acetone fixed prior to storage at −70°C. Sections were stained with a primary antibody for 1 h (at room temperature) against cell-type-specific antigens: anti-glial fibrillary acidic protein (GFAP) (rabbit anti-cow GFAP; Dako, Carpinteria, Calif.) to identify astrocytes, F4/80 to identify microglia/macrophages, and antigalactocerebroside (rabbit antigalactocerebroside; Chemicon International, Los Angeles, Calif.) to identify oligodendroglia. Neurons were identified by their typical morphology. The sections were then washed with PBS and incubated with a specific secondary antibody (fluorescein isothiocyanate-conjugated immunoglobulin G Fc and goat anti-rat goat anti-rabbit immunoglobulin G; Chemicon) for 30 min. After a PBS wash, the sections were stained with the anti-JHMV MAb J.3.3 for 1 h. This was followed by a PBS wash and then incubation with goat anti-mouse immunoglobulin G Cy3 (Chemicon) for 30 min. The sections were washed with PBS, mounted with Vecta Shield mounting medium (Vector Laboratories), and then viewed with a Zeiss confocal laser-scanning microscope (model LSM2; Carl Zeiss, Thornwood, N.Y.).

TABLE 1. Protection following vaccination with N protein

Immunization[a]	Titer[b]	Route	No. live/total[c]	% Protection
JHMV	10^6	i.p.	10/10	100
UV-inactivated vJN455		i.p.	6/7	86
UV-inactivated vSC8		i.p.	0/6	0
vJN455	2×10^7	i.p.	9/13	69
vJN455	2×10^7	i.v.	2/5	40
vJN374	10^8	i.v.	1/4	25
vJN51	2×10^7	i.p.	0/4	0
vSC8	2×10^7	i.p.	0/19	0

[a] Mice were immunized 21 days prior to i.c. challenge with 100 PFU of JHMV.
[b] Titer of virus used for immunization.
[c] Determined 21 days following challenge.

RESULTS

Protection via vaccination. Protection from coronavirus infection following vaccination with vectors expressing the N protein has varied significantly (18, 53, 57). Immunization of BALB/c mice with JHMV or a UV-inactivated preparation containing the JHMV N protein, but not similar UV-inactivated lysates prepared from control rVV (vSC8)-infected cells, protects from JHMV rechallenge (Table 1). By contrast, immunization with rVV expressing the N protein of JHMV (vJN455) or a carboxy-terminal truncation (vJN374) lacking the carboxy terminal MAb site (44) was only partially protective (Table 1). Reduced protection following vJN374 vaccination could indicate that antibodies specific for the acidic carboxy-terminal portion of the N protein (44) are important in protective immunity (29, 33). Vaccination with vJN51, which expresses amino acids 301 to 351 of the N protein, was also unable to protect. This sequence contains the previously defined N-protein CTL epitope (amino acids 318 to 326) (5). These data indicated that the peripheral induction of an immune response, including N-protein-specific CTL, by using rVV vectors was not capable of fully protecting mice from a lethal JHMV challenge and that $CD4^+$ T cells and antibody specific for the N protein contribute to protection (23, 29, 33).

CTL protect from Acute JHMV infection. Analysis of the $CD8^+$ T cells within the CNS of JHMV-infected mice showed a high frequency of N-protein-specific CTL compared with JHMV-specific CTL restricted to a nonstructural protein(s) (48). These data suggested that either rapid recruitment or local expansion of N-protein-specific CTL within the CNS was critical in the pathogenesis of JHMV. CTL specific for the L^d-restricted epitope within the JHMV N protein were derived from spleen cells of JHMV-immunized mice and expanded in vitro by incubation with 1 μM pN318-335 peptide as previously described (5, 6). Effectors were tested for cytolytic activity on target cells expressing a carboxy-truncated N protein (vJN374), which contains the CTL epitope but lacks the carboxy-terminal

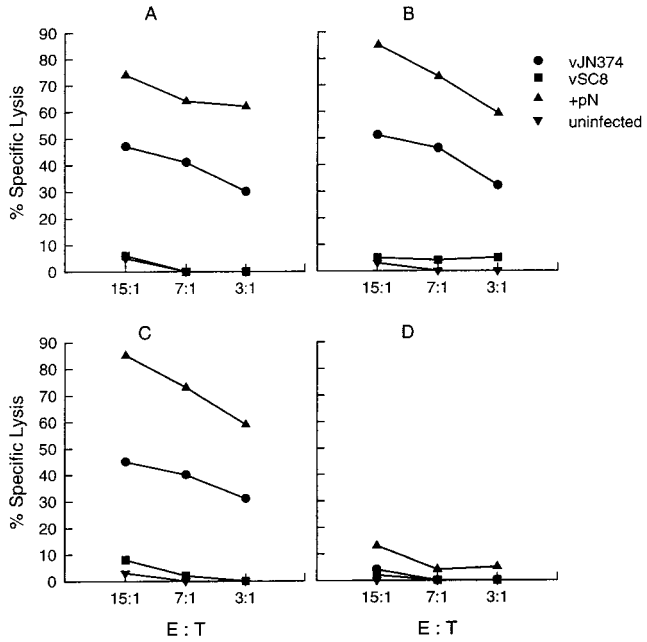


FIG. 1. CD8$^+$ cells express anti-N-protein cytolytic activity. Spleen cells from JHMV-immunized mice were cultured for 6 days in the presence of 1 μM pN318-335 peptide. CTL activity was tested on uninfected targets (▼), pN318-335 peptide-coated targets (▲), or targets infected with either vJN374 (●) or control vSC8 (■). Effectors were either untreated (A) or incubated with complement only (B), anti-CD4 MAb plus complement (C), or anti-CD8 plus complement (D). The effector/target (E:T) ratio is shown on the *x* axis.

J.3.3 MAb epitope, target cells infected with the control rVV expressing the *E. coli lacZ* gene (vSC8), and pN318-335-peptide treated and untreated target cells. Figure 1 shows that CTL specifically lysed target cells coated with peptide or infected with vJN374 but not uninfected (without peptide) or vSC8-infected target cells. The identity of the effector population was tested following complement-mediated depletion of T-cell subsets (47, 48). Treatment with anti-CD4 plus complement or complement only had no effect on recognition of N-protein-expressing target cells. By contrast, treatment of the effectors with anti-CD8 MAb plus complement eliminated specific lysis (Fig. 1). The CD8$^+$ effector population recognizes JHMV-infected target cells but not target cells expressing any of the other virus structural proteins (data not shown). These data demonstrate that this effector population contains a CD8$^+$ CTL population which specifically recognizes the N epitope.

Effectors from JHMV-immunized mice stimulated in vitro with peptide were transferred to syngeneic BALB/c mice, using three routes to determine if N-protein-specific CTL could protect JHMV-infected mice from acute disease. Similar to results of previous studies analyzing the adoptive transfer of CD4$^+$ T cells (23, 49, 62), the adoptive transfer of 10^6 CTL i.c. protected all mice challenged with JHMV (Table 2). The i.c. transfer of an N-protein-specific CTL clone (10^6 per recipient) also protected from a lethal JHMV infection (data not shown). No mice were protected following the transfer of 5×10^5 CTL i.c. Intravenous (i.v.) adoptive transfer was less efficient than the direct transfer i.c., requiring at least 10^7 cells to provide complete protection. By contrast, the transfer of 2×10^7 cells i.p. was not able to protect mice from a subsequent lethal challenge with JHMV. The reduced efficiency of CTL transferred by peripheral routes to protect from a subsequent lethal JHMV challenge suggests that these CTL may be unable to traffic efficiently into the CNS (2, 3, 21) and/or that the number of effectors entering the CNS is insufficient to mediate protection.

TABLE 2. Protection mediated by JHMV-specific CTL

Route	No. of cells transferred	No. of live/total[a]
i.c.	10^6	9/9
i.c.	5×10^5	0/4
i.v.	5×10^7	4/4
i.v.	2×10^7	10/10
i.v.	10^7	6/6
i.v.	5×10^6	0/4
i.p.	2×10^7	0/3

[a] Determined 21 days following challenge with 100 PFU of JHMV within 6 h of transfer.

CTL reduce virus titer within the CNS. These data indicated that the N-protein-specific CTL protect mice from a lethal JHMV challenge; however, the transfer of JHMV-specific CD4$^+$ T cells can also protect mice without significantly reducing the titer of virus within the CNS (23, 49, 62). Virus titers in the brains of untreated JHMV-infected mice were therefore compared with those in CTL recipients at 3, 5, and 7 days p.i. to determine if the CTL were providing protection by a direct reduction in virus replication. At all three days tested, the i.v. adoptive transfer of 10^7 CTL effectively reduced the titer of virus within the CNS (Table 3). Further, the transfer of 2.5×10^7 cells was more effective than the transfer of 10^7 cells, consistent with the dose-dependent protection (Table 2), indicating that the JHMV-specific CD8$^+$ CTL population protects via a reduction of infectious virus within the CNS.

CTL induced by immunization with N-protein peptides. The transferred cell population derived from the secondary in vitro stimulation of spleen cells from JHMV-immunized mice with peptide contains approximately 97% Thy-1$^+$, 80% CD8$^+$, and 20% CD4$^+$ T cells, determined by flow cytometry (data not shown). To initially examine the possibility that an antibody or CD4-mediated immune response participated in protection from fatal JHMV infection, CTL were derived from mice immunized with rVV which expresses amino acids 301 to 351 of the N protein (vJN51) and cultured for 6 days with 1 μM pN318-335 peptide. Table 4 shows that transfer of effectors derived from vJN51-immunized donors protects mice from a lethal challenge with JHMV and reduces virus titer within the CNS of JHMV-infected mice. By contrast, the transfer of human immunodeficiency virus gp160-specific CTL was unable to either protect mice (data not shown) or reduce virus titer in the CNS (Table 4). No N-protein-specific CTL are induced from JHMV-immunized mice stimulated with the heterologous

TABLE 3. Effect of JHMV-specific CTL on CNS virus replication

Day	Immunogen	No. of cells transferred[a]	Virus titer (PFU/g of brain)
3	None	None	4.1×10^5
	JHMV	10^7	9.6×10^3
5	None	None	4.1×10^6
	JHMV	10^7	1.0×10^5
	JHMV	2.5×10^7	5.3×10^3
7	None	None	1.5×10^4
	JHMV	10^7	4.5×10^2

[a] Spleen cells from JHMV-immunized donors stimulated for 6 days on pN318-335; viable cells isolated by using Lympholyte M prior to i.v. transfer.

TABLE 4. CTL from mice immunized with N-protein-expressing rVV[a]

Immunogen	No. of cells transferred	No. live/ total[b]	Virus titer (PFU/g of brain)[c]
None	None	0/6	4.1×10^6
vJN51	10^7	3/3	1.5×10^5
None	None	0/5	2.2×10^6
vJN9	10^7	4/4	7.4×10^5
None	None	ND[d]	2.9×10^5
vPE16	10^7	ND	9.2×10^5

[a] Spleen cells from immunized mice were stimulated with pN (vJN51 or vJN9) or p18 (vPE16) peptide for 6 days. Viable cells were isolated by using Lympholyte M and transferred i.v. Mice were challenged i.c. with 100 PFU of JHMV within 6 h of T-cell transfer.
[b] Determined at 21 days postchallenge.
[c] Determined 5 days postchallenge.
[d] ND, not done.

gp160 (p18) peptide, nor are N-protein-specific CTL induced by incubation in the presence of pN318-335 peptide without prior JHMV immunization (6).

We have recently mapped the domains recognized by MAbs specific for the N protein and determined that none recognized an epitope contained within the region encoded by vJN51 (44). However, preliminary data suggest the presence of $CD4^+$ T-cell epitopes amino terminal to the pN318-326 CTL epitope (50), whose precise location has not been determined. To rule out the involvement of other epitopes, effectors were derived from mice immunized with vJN9, which expresses only the nine amino acids (318 to 326) comprising the optimal N-protein epitope. Effectors derived from vJN9-immunized mice and stimulated for 6 days with pN318-335 peptide were also protective but were less efficient at reducing virus in the CNS of recipients than the effectors derived from vJN51-immunized mice (Table 4). Direct transfer of 10^6 vJN9-derived effectors into the CNS prior to JHMV infection was very effective at reducing virus replication (data not shown), consistent with the protection mediated by the similar transfer of JHMV-induced effectors (Table 2).

CTL derived following immunization with vJN9 were also tested for cytolytic activity. These effectors specifically recognized target cells infected with vJN374 and coated with pN318-335 peptide (Fig. 2). Further, Fig. 2 shows that the cytolytic activity is sensitive to anti-CD8-plus-complement treatment but not to treatment with complement only or anti-CD4 plus complement. Similar data were obtained for effectors derived from vJN51-immunized mice (data not shown). The cytolytic activity of CTL obtained from the spleen cells of vJN9-immunized mice was lower than the activity obtained from JHMV-immunized mice stimulated under identical in vitro conditions (Fig. 1). The reduced ability of effectors derived from vJN9-immunized mice to clear virus from the CNS following i.v. transfer may reflect limited CTL induction in the absence of associated $CD4^+$ helper epitopes (37) and/or direct participation of $CD4^+$ T cells.

Protection and virus reduction in vivo is class I specific. The CTL response to the N epitope in BALB/c mice is restricted to the MHC class I L^d molecule (5, 58). To further demonstrate that the reduction in virus titer within the CNS is mediated by an MHC class I-restricted effector population, the ability of effectors derived from JHMV-immunized donors to affect the virus titer within the CNS of B10.D2 (K^d, D^d, L^d), B10.A(5R) (K^b, D^d, L^d), and C57BL/10 (K^b, D^b) mice infected with JHMV was examined. Table 5 shows that following the transfer of BALB/c-derived CTL, virus titer was reduced only slightly in

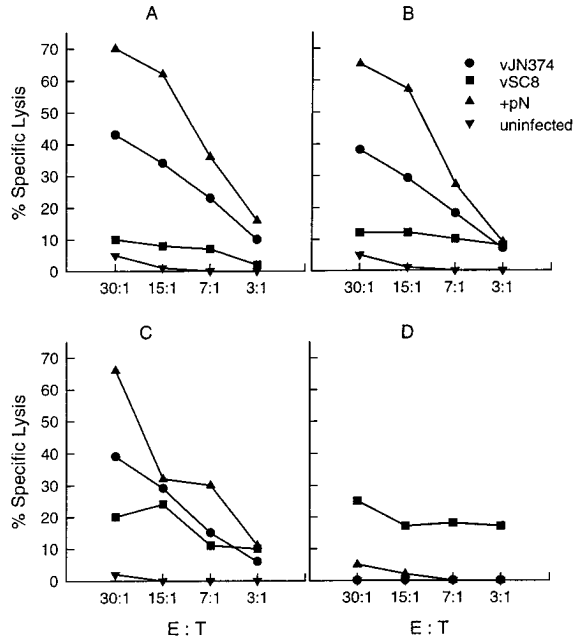


FIG. 2. Cytolytic activity of effector cells derived from vJN9-immunized mice. Spleen cells were cultured for 6 days in the presence of 1 μM pN318-335 peptide. CTL activity was tested on uninfected targets (▼), pN318-335 peptide-coated targets (▲), or targets infected with either vJN374 (●) or control vSC8 (■). Effectors were either untreated (A) or incubated with complement only (B), anti-CD4 MAb plus complement (C), or anti-CD8 plus complement (D). The effector/target (E:T) ratio is shown on the *x* axis.

C57BL/10 mice. By contrast, the adoptive transfer of these cells into both the *H-2*^{d} B10.D2 mice and the partially compatible B10.A(5R) mice resulted in a reduction of virus within the CNS. These data confirm the primary role of the MHC class I-restricted CTL population in the reduction of virus with the CNS of JHMV-infected mice.

Histological analysis of protection. JHMV replicates within all cells of the CNS, although it is primarily tropic for oligodendroglia (16, 26, 27, 56). Thus, cytolytic infection of oligodendroglia is thought to be responsible for JHMV-induced primary demyelination. The inability of N-protein-specific CTL administered i.v. to completely clear virus from the CNS suggested that potential target cells which do not express MHC class I in situ may be viral reservoirs. By histopathological analysis, brains and spinal cords from mice given 10^7 N-protein-specific CTL derived from JHMV-immunized mice were compared with those of control mice at 3, 5, and 7 days p.i. During acute infection, viral antigen was detected in oligodendroglia, astrocytes, microglia, ependymal cells, perivascular

TABLE 5. Virus reduction is controlled by MHC class I

Recipients	Haplotype	No. of cells transferred[a]	Virus titer (PFU/g of brain)
C57BL/10	K^b, $I\text{-}A^b$, D^b, L^b	None	1.8×10^4
		2×10^7	1.1×10^4
B10.D2	K^d, $I\text{-}A^d$, D^d, L^d	None	3.3×10^4
		2×10^7	6.3×10^3
B10.A(5R)	K^b, $I\text{-}A^b$, D^d, L^d	None	2.0×10^4
		2×10^7	3.6×10^1

[a] Spleen cells from JHMV-immunized BALB/c (*H-2*^{d}) mice were stimulated for 6 days with pN peptide and purified by using Lympholyte M prior to transfer. Recipients were challenged i.c. within 6 h of transfer, using 100 PFU of JHMV.

macrophages, and neurons, accompanied by a vigorous panencephalomyelitis (Fig. 3). Comparison of recipients with untreated control mice clearly showed that virus antigen was reduced in CTL recipients. The morphology of virus-positive cells in recipients suggested that viral antigen was reduced within all cell types; however, there was only marginal reduction in virus-infected oligodendroglia (Fig. 3). Double-staining experiments revealed only small numbers of astrocytes ($GFAP^+$) and microglia/macrophages ($F4/80^+$) which colocalized with virus; however, many of virus-positive cells colocalized with the galactocerebroside oligodendroglial marker (data not shown). These results suggest that the N-protein-specific CTL reduce virus infection in CNS cells capable of expressing MHC class I molecules (28). The reduction of viral antigen in neurons following CTL immunotherapy cannot be directly attributed to a cytolytic attack on these MHC class I-negative cells (20, 28, 34). This may reflect an overall reduction in infectious virus, coupled with the reduced tropism of JHMV for neurons, or possibly the ability of cytokines to modify neuronal infection (16, 26). These data clearly indicate that CTL specific for the N protein play a crucial role in reducing virus replication and the extent of primary demyelination via the recognition of the N-protein epitope presented on the surface of MHC class I-positive cells. Only minimal reductions in antigen-positive oligodendroglia were detected. Whether this finding reflects a differential ability of cytokines to protect oligodendroglia versus neurons or represents an early restriction in virus replication preventing local spread is not clear.

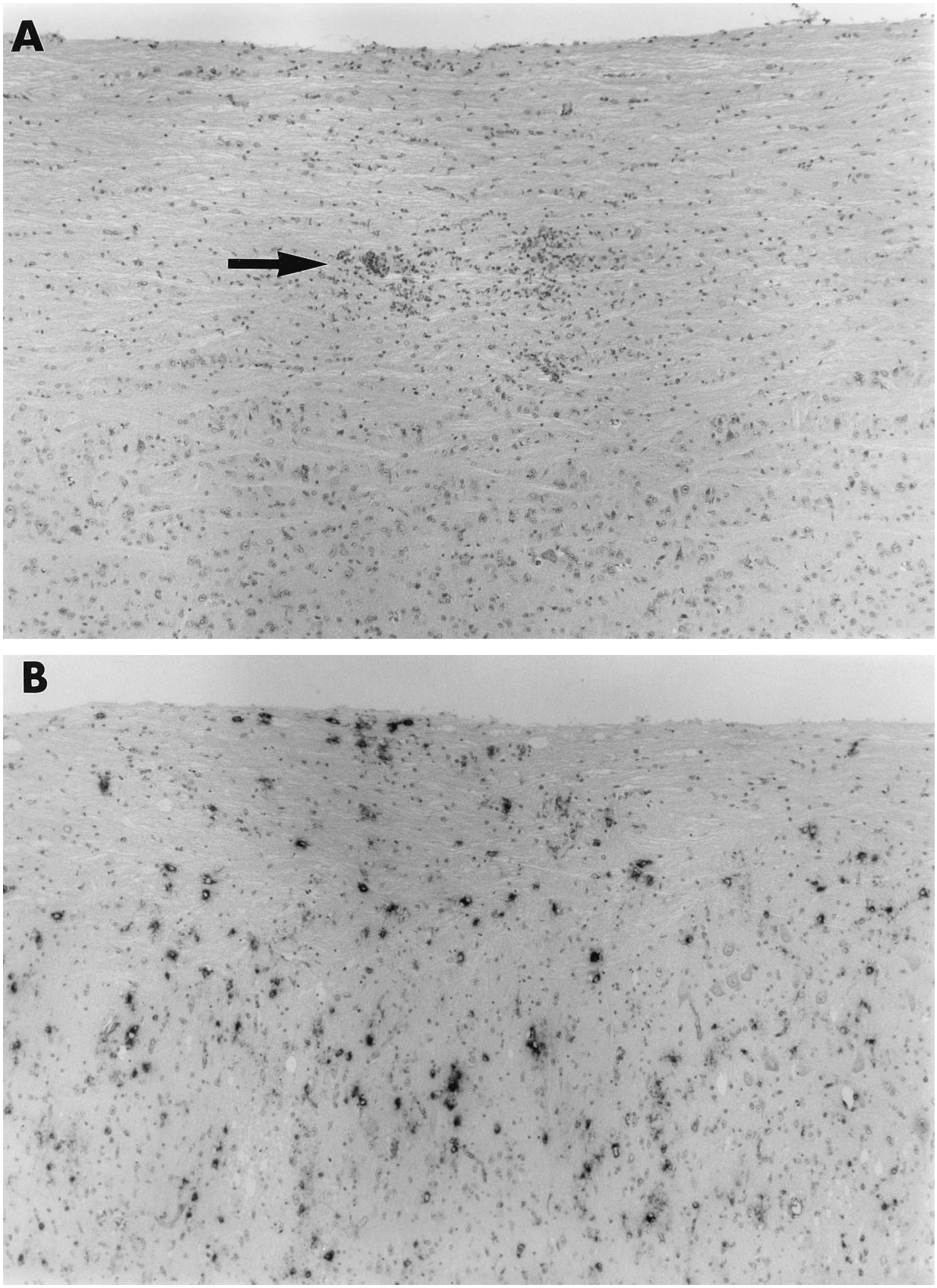
To determine the long-term effects of partial protection afforded by the adoptive transfer of CTL, surviving mice were examined for the presence of mononuclear cell infiltrates, demyelination, and persistent viral antigen at 14, 30, 60, and 90 days p.i. Although occasional antigen-positive cells were found within the spinal cords of these mice, no evidence of chronic ongoing demyelination was detected (data not shown). These findings contrast with histological analysis of mice protected from acute disease with either nonneutralizing MAb or virus-specific $CD4^+$ T cells, which show evidence of chronic demyelination associated with persistence of viral antigen (14–16, 26, 51).

DISCUSSION

JHMV infection of the CNS results in an acute encephalomyelitis with primary demyelination and is associated with a chronic demyelinating disease in survivors (11, 16, 26, 27, 51, 56). Virus appears to persist in chronically infected survivors in a noninfectious state, primarily in astrocytes (26, 35, 51); however, the mechanism by which persistent virus infection of astrocytes results in chronic ongoing primary demyelination is not clear. A variety of studies indicate that protective immune responses are a critical component in and establishment of persistent CNS infection (26). Protective immune responses participate in the resolution of acute JHMV infection of the CNS. In contrast to other viruses in which single protective mechanisms predominate, the transfer of both MAbs which do not neutralize virus in vitro and $CD4^+$ T cells protects recipients from a subsequent lethal JHMV challenge (17, 23, 49, 62). Protection by these immunotherapies is not associated with a significant reduction of virus replication within the CNS. There also appears to be increased destruction of oligodendroglia with an increase in the chronic form of JHMV-induced recurrent demyelination, possibly due to the inability of these immune effectors to reduce infection in oligodendrocytes (15, 26). Both the transfer of $CD4^+$ T cells and the transfer of an uncharacterized clonal population of K^d-restricted JHMV-specific CTL also protect from a lethal challenge at least partially mediated by a reduction of virus replication in the CNS (55, 62).

Immunity induced by immunization with viral nucleocapsid proteins comprises not only an antibody response, which may in some cases be protective on its own (29, 33), but also $CD4^+$ and $CD8^+$ T-cell responses. For example, immunization with purified influenza virus NP protects mice, whereas immunization with rVV expressing the NP protein does not (43, 61). Immunization with an rVV expressing the measles virus N protein provides protection; however, it was not mediated by $CD8^+$ T cells (9). By contrast, immunization with an rVV expressing the N protein of lymphocytic choriomeningitis virus induces a $CD8^+$ T-cell-mediated protection (19). Interestingly, immunization of rats with an rVV expressing the JHMV N protein was unable to confer protection; however, expression of the MHV N protein by using an adenovirus vector did confer protection to mice (18, 57). Vaccination with an rVV expressing the JHMV N protein (vJN455) or truncated N protein (vJN374) lacking the carboxy-terminal domain containing an epitope for a MAb specific for the N protein which can protect following passive transfer (50) was able to confer only partial protection to subsequent JHMV challenge. By contrast, immunization with a UV-inactivated lysate prepared from vJN455-infected cells conferred greater protection than immunization with live vJN455. Reduced protection following immunization with vJN374 vaccination could indicate that the acidic carboxy-terminal portion of the N protein is important for a protective immunity. The possibility that antibody is involved is supported by the observation that MAb J.3.3, specific for the carboxy terminus of the N protein, can protect mice from a lethal infection, while MAb J.3.5, specific for a more carboxy epitope (amino acids 249 to 277) (44), is unable to provide protection (50). Since the epitope recognized by MAb J.3.3, but not the epitope recognized by MAb J.3.5, is expressed on the surface of infected cells (25), it is plausible that cell surface expression of this epitope contributes to the protection mediated by anti-N-protein MAb (29, 33). Finally, vaccination with an rVV (vJN51) expressing a 51-residue peptide including the CTL epitope (5) was not able to confer protection. These data suggest that either antibody or $CD4^+$ effectors, or both, induced peripherally via immunization with an rVV expressing the N protein may provide partial protection, while the peripheral induction of CTL is not fully protective. These data are consistent with our inability to detect N-protein-specific CTL in the periphery during acute JHMV infection (48).

FIG. 3. Paraffin sections of spinal cord from infected control and CTL recipient mice at 5 days p.i. (A) Acute JHMV infection is accompanied by encephalomyelitis with prominent perivascular cuffing (arrows) by mononuclear cells. Hematoxylin and eosin; magnification, 125×. (B) Immunoperoxidase stain for JHMV in infected control animals. Numerous immunoreactive cells are identified, predominantly in the white matter. Hematoxylin counterstain; magnification, 125×. (C) At higher magnification, the immunoreactive cells have the morphology of various glial cells and macrophages. Hematoxylin counterstain; magnification, 500×. (D) Immunoperoxidase stain for JHMV in infected CTL-recipient mice. Only a small number of immunoreactive cells (arrows) are identified. Hematoxylin counterstain; magnification, 125×. (E and F) At higher magnification, the immunoreactive cells have the morphology of oligodendroglia. Hematoxylin counterstain; magnification, 500×.


A
B

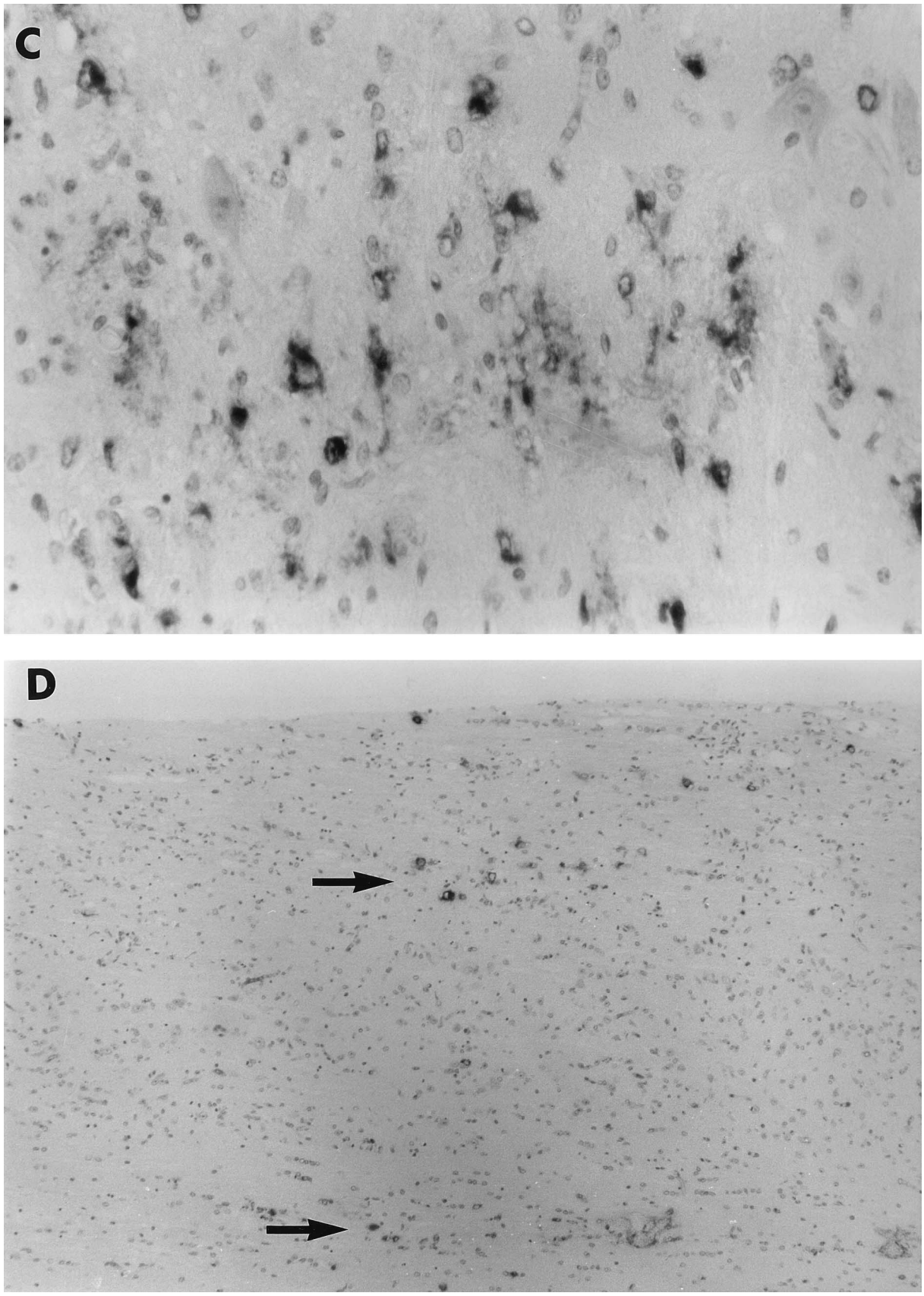


FIG. 3—*Continued.*

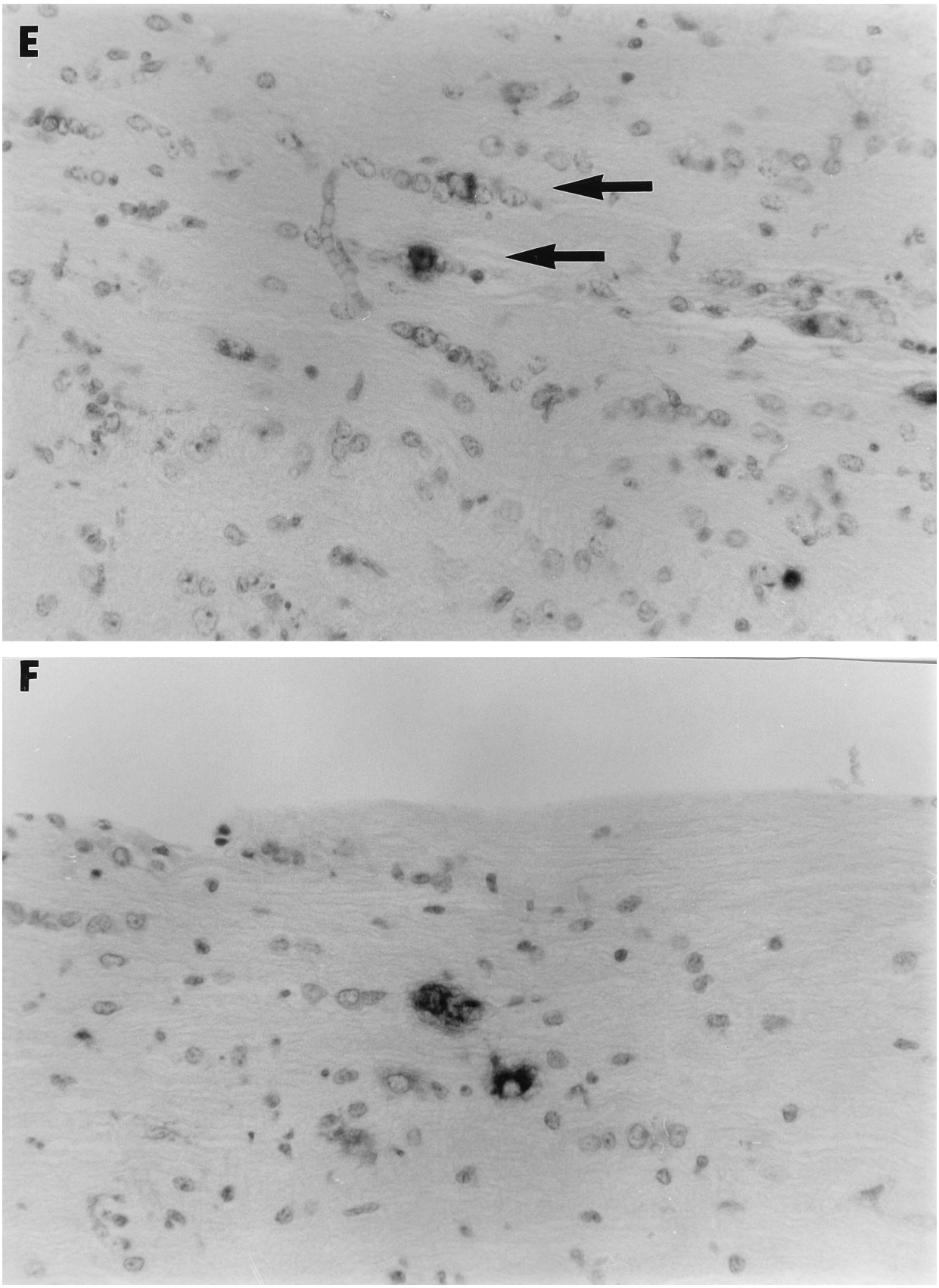


FIG. 3—*Continued.*

In this report, we examined the potential for the CTL specific for the L^d-restricted CTL epitope within the N protein (residues 318 to 326) to protect mice from lethal JHMV challenge via adoptive transfer and attempted to define the mechanism of this protection. The data indicate that CTL specific for this epitope can protect mice from a lethal challenge following both direct transfer into the CNS and i.v. transfer. Transfer of cells into the peritoneal cavity was unable to protect, although transfer of N-protein-specific $CD4^+$ T cells by this route has previously been shown to protect (23). Consistent with nondetectable N-protein-specific CTL in the peripheral lymphoid organs following infection (48), the increased efficiency of direct transfer may reflect the decreased ability of $CD8^+$ T cells to gain entry into the CNS during JHMV infection. Further, our analysis of protection mediated by CTL induced via immunization with an rVV expressing only the nine-residue epitope showed that these cells were less efficient at recognition of target cells in vitro (Fig. 2) and less efficient at reducing virus within the CNS (Table 4). This difference may be related to the reduced induction of CTL effectors when not coupled to a CD4 Th epitope (38). Previous studies have indicated a requirement for $CD4^+$ T-cell-mediated help in the induction of JHMV-specific CTL (23, 52, 58). Alternatively, reduced protection may be related to variable expression of adhesion molecules or secretion of cytokines, both of which have recently been implicated in the access of T cells into the CNS (2, 21, 23, 30, 37).

Incomplete reduction of virus within the CNS suggested either that the cells were unable to traffic efficiently into the CNS (3) or that not all infected cells express MHC class I. The expression of class I during infection of the CNS is predominantly confined to astrocytes and microglia (1, 28), both of which are infected by JHMV (27, 28, 60); however, in addition to these cells, neurons are also infected, although the oligodendroglia appear to be a major target of virus infection (27, 56). Neither class I nor class II expression on oligodendroglia has been reported in vivo (4, 28, 36), although these cells can express class II in vitro following cytokine treatment (4, 8). Histological analysis of the distribution of viral antigen in the CNS of untreated mice and recipients of $CD8^+$ T cells demonstrated a reduction in viral antigen in the recipients, consistent with the reduction in infectious virus. By contrast, protection was associated with a decrease in viral antigen from cells which have both the morphology and the distribution consistent with astrocytes and microglia. There was little reduction of antigen in oligodendroglia during the acute phase of infection; however, analysis of survivors showed a paucity of infected cells and no evidence of ongoing demyelination. The absence of detectable demyelination in these mice is not surprising, given the limited number of cells with persistent antigen and the focal nature of recurrent demyelination in mice chronically infected with JHMV (14).

The polyclonal nature of the immune response to JHMV infection of the CNS may represent a feature of organ-specific control. It is likely that infection of the CNS results in a broad immune response, employing a variety of effector mechanisms, to efficiently clear infection prior to the induction of pathological changes associated with nonspecific tissue destruction (39). Alternatively, in JHMV infection of the CNS, a vigorous immune response designed to reduce tissue destruction appears ineffective at completely clearing virus from the CNS and may contribute to the persistent infection and associated chronic ongoing demyelination. The low turnover rate and regenerative capacity of CNS cells suggest that destruction of a small number of cells could have long-term detrimental effects on the host, similar to sequelae seen following measles virus-induced encephalitis in humans (13). The ability of N-protein-specific CTL to reduce the levels of infectious virus within the CNS confirms previous data suggesting that CTL are critical in the resolution of acute viral encephalomyelitis (52, 58, 62). However, the precise mechanism(s) limiting virus replication is not clear. The adoptive transfer of a K^d-restricted CTL directly into the CNS of JHMV-infected mice suggested that protection was associated with increased tissue destruction (62). Whether these cells directly reduce virus within the CNS by lysis of virus-infected target cells, by the release of cytokines, or by a combination of effects is unclear. The release of cytokines from $CD8^+$ CTL has previously been implicated in the protection (24, 37). Although injection of JHMV-infected mice with recombinant gamma interferon (IFN-γ) had little or no effect on virus replication within the CNS, the passive transfer of an anti-INF-γ MAb did increase virus replication in peripheral organs (40). Numerous cells, including natural killer (NK) cells and $CD4^+$ and $CD8^+$ T cells, can secrete IFN-γ. Although there is no evidence for a role of NK cells in JHMV infection (45), NK cells are rapidly recruited into the CNS during JHMV infection (59). In addition, both $CD4^+$ and $CD8^+$ T cells are critical for protection from JHMV (11, 18, 26). Therefore the role of IFN-γ as a contributor to the observed $CD8^+$ T-cell-mediated virus reduction in the CNS is unclear.

The data contained in this report are the first demonstration that $CD8^+$ CTL specific for a defined JHMV epitope reduce the replication of virus within the CNS of infected mice, and the data are consistent with rapid recruitment or expansion within the CNS following JHMV infection (12, 48, 59). Further, the data show that these CTL, although effective at recognition of target cells in vitro, are unable to completely clear infectious virus from the CNS. Consistent with a class I-mediated effect, the reduction of viral antigen was maximal from cell types within the CNS which express MHC class I (1, 28), with only little reduction in MHC class I-negative oligodendroglia. This finding suggests that CTL specific for JHMV are unable to recognize antigen associated with these cells, thus implicating the recently described $CD4^+$ T cells with antiviral (48) cytolytic activity in clearance of virus from oligodendroglia. In addition, our data suggest that the reduction of viral antigen associated with neurons may reflect reduced virus replication in conjunction with a low affinity of JHMV for neurons (26, 27). Although the expression of cytokine receptors expressed by oligodendroglia and neurons in situ has not been examined, our data suggest that local production of cytokines is not a major effector mechanism for viral clearance. Conversely, histological evidence indicates that CD8-mediated reduction of viral antigen was associated with only class I-positive cells within the CNS and is consistent with increased MHC class I in the CNS following viral infection (1). Finally, these data suggest that CTL-mediated immunotherapy results in diminished chronic demyelination. It is, however, not clear whether this represents an overall reduction in the number of infected oligodendroglia or the absence of persistently infected astrocytes, previously shown to correlate with JHMV persistence in the CNS (35).

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