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Mouse Hepatitis Virus Is Cleared from the Central Nervous Systems of Mice Lacking Perforin-Mediated Cytolysis

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Perforin-deficient [perforin (−/−)] mice were infected with two strains of JHM virus (JHMV) to analyze the role of perforin-mediated cytotoxicity in acute lethal and subacute central nervous system (CNS) infections. During both acute and subacute infections, the overall mortality of the perforin (−/−) mice was not different from that of the controls. Perforin (−/−) mice survived longer than the controls, consistent with reduced morbidity. Both strains of virus were cleared from the perforin (−/−) mice as in the controls; however, the rate of clearance was delayed in the perforin (−/−) mice, indicating that perforin-mediated cytolysis is involved in viral clearance. The absence of perforin-mediated cytolysis did not prevent encephalomyelitis or extensive demyelination. Cells undergoing apoptosis were detected in the CNS of both the perforin (−/−) and control groups, indicating that perforin is not essential for programmed cell death. Neutralizing antibodies were not detected in either group of mice until day 9 postinfection, when the majority of the virus had been cleared. These data further confirm the importance of cell-mediated cytotoxicity and suggest that additional components of the immune response contribute to the clearance of JHMV from the CNS.

Infection of the central nervous system (CNS) with JHM virus (JHMV), a neurotropic member of the mouse hepatitis virus (MHV) group of coronaviruses, induces acute encephalomyelitis with primary demyelination in both rodents and primates (29, 34, 49). Mice which survive develop a persistent CNS infection characterized by persistence of viral antigen with rarely recoverable infectious virus (28). The persistent infection is also accompanied by chronic ongoing demyelination (39). Although demyelination during JHMV infection was attributed to direct viral cytolysis of oligodendroglia (29), recent evidence analyzing immunosuppressed mice has clearly shown that demyelination is associated with a virus-induced immune response (20, 48). JHMV infection elicits cytotoxic T-lymphocyte (CTL), natural killer (NK) cell, $CD4^+$ T-cell, and B-cell infiltration into the CNS (10, 52) as well as antiviral antibody responses; however, the precise nature of the protective response and the immune mechanism(s) leading to viral clearance and immune-mediated demyelination are still undefined (28). Contrary to other viral infections where a major effector mechanism dominates immunopathogenesis (4, 31, 33), survival, viral clearance, and subsequent development of a persistent infection associated with chronic demyelination following a JHMV infection appear to be influenced by both cellular and humoral immune responses (28). Because the recruitment of T cells into the infected CNS correlates with viral clearance and occurs prior to detectable serum neutralizing antibodies (10, 52), emphasis has been placed on understanding the role of cell-mediated immunity in JHMV pathogenesis. Recently, CTL epitopes encoded within the JHMV structural proteins have been characterized in both C57BL/6 ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) mice (1, 2, 7, 42). Consistent with the concept that $CD8^+$ CTL are critical in viral clearance during JHMV infection (46, 51), adoptive transfer of JHMV-specific $CD8^+$ CTL provides protection from both acute and chronic infection by limiting viral replication in major histocompatibility complex class I-positive astrocytes and microglia but not oligodendroglia (44). However, in addition to the protective role for $CD8^+$ CTL, the adoptive transfer of some but not all JHMV-specific $CD4^+$ T cells also results in clearance of virus from the CNS (26, 41, 50, 53). These data suggest that cell-mediated cytotoxicity, either by $CD8^+$ CTL or possibly by a contribution from the $CD4^+$ T-cell population, is an important aspect of JHMV-induced CNS disease.

Cell-mediated cytotoxicity is predominantly mediated via two independent pathways: a granule exocytosis model involving perforin, a complement-like protein, and Fas ligand (FasL) (3, 16, 23, 32). It has been suggested that the predominant effector mechanisms utilized in an antiviral protective response correlate with the ability of the virus to induce cellular cytopathogenicity (24, 25, 55). For example, immunity to lymphocytic choriomeningitis virus (LCMV), a noncytopathic virus, is exclusively mediated by the $CD8^+$ CTL response and is perforin-dependent (22). By contrast, protection from cytopathic viruses, i.e., vaccinia virus, vesicular stomatitis virus, and Semliki Forest virus, is mediated by noncytolytic effectors (24, 25). In vitro, JHMV produces rapid cytopathology accompanied by cell-cell fusion and cell death. Although MHV infection in vivo has been associated with giant cell formation within the gastrointestinal system (9), only few reports indicate giant cell formation or direct cytopathogenicity following JHMV infection of the CNS (15, 18, 29). Therefore, JHMV infection in vivo cannot be readily classified as either cytopathic or noncytopathic, consistent with the potential role for a variety of immune effectors to participate in both viral clearance from the CNS and primary demyelination (28). In this study, mice deficient in the perforin gene [perforin (−/−) mice] were used to examine the role of cell-mediated perforin-dependent cytotoxicity in viral clearance and demyelination following JHMV infection of the CNS.

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MATERIALS AND METHODS

Virus and cell lines. The DM isolate of JHMV was propagated and plaque assayed by using the murine DBT astrocytoma cell line as previously described

(40). DM is a plaque-purified isolate with plaque morphology and pathogenesis identical to those of parental JHMV (39, 49). Mice were infected by intracerebral (i.c.) inoculation with 100 PFU contained in a volume of 32 μl which results in a uniformly fatal encephalomyelitis. In some experiments, mice were infected i.c. with 50 PFU of the 2.2v-1 monoclonal antibody (MAb)-derived variant of JHMV (12). The 2.2v-1 virus, kindly supplied by J. Fleming (University of Wisconsin), produces an attenuated infection with severe demyelinating disease (12) and was tested for reversion prior to use. The (*H-2*^{b})-derived MC57G cell line was obtained from the American Type Culture Collection and maintained as previously described (2).

JHMV replication in the CNS was determined by plaque assay as previously described (41, 44, 45). Briefly, one half of the brains was homogenized in 2.0 ml of Dulbecco's phosphate-buffered saline (pH 7.4), using Ten Broeck tissue homogenizers. The other half was processed for histopathology (see below). Following centrifugation at 200 × *g* for 7 min at 4°C, supernatants were collected and either assayed immediately or frozen at −70°C. Homogenates were serially diluted in serum-free minimum essential medium (MEM) containing 10% tryptose phosphate broth. DBT cells, grown to confluence in 60-mm-diameter plates, were washed twice with MEM before virus adsorption (0.2 ml/plate) for 90 min at room temperature. After adsorption, 5 ml of MEM supplemented with 10% tryptose phosphate broth, 0.6% agarose, and 1% penicillin-streptomycin-amphotericin B (Fungizone) solution (JRH Biosciences) was added to each plate. Plates were incubated at 37°C, and the number of plaques was determined after 24 h (DM) or 48 h (2.2v-1). Data presented are the average of triplicate determinations for groups of three or more mice.

Mice. Seven- to eight-week-old male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Sex- and age-matched perforin (−/−) and perforin (+/+) control mice were kindly provided by W. Clark (University of California Los Angeles). Perforin (+/+) and perforin (−/−) 129 × C57BL/6 mice derived as previously described (47) were crossed once with C57BL/6 mice and maintained by intercrossing. All mice were seronegative for MHV. In some experiments, mice were immunosuppressed with 800 rads of γ-irradiation on the same day as infection.

Clinical scores. Infected mice were graded for severity of clinical disease as previously described (13): 0, healthy; 1, ruffled fur and hunched back; 2, slow mobility and inability to return to upright position when turned on the back; 3, paralysis and wasting; 4, moribund and death. Average clinical scores for at least three mice per group are reported.

Induction of effectors and cytotoxic assay. Spleen cell suspensions were prepared from either C57BL/6 or perforin (−/−) mice immunized 3 to 6 weeks earlier by intraperitoneal infection of 2×10^6 PFU of JHMV as previously described (2). Spleen cells (10^8) from immunized 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 (2). CTL were stimulated by in vitro inclusion of 1 μM peptide comprising amino acids 510 to 518 of the amino-terminal portion of the S protein (S 510-518 epitope), a previously described major CTL epitope in *H-2*^{b} mice (2, 7).

Cytolytic activity was measured as previously described (42). In vitro-stimulated spleen cells were added at various ratios to round-bottom 96-well plates (Falcon Plastics, Oxnard, Calif.) in 100 μl of RPMI complete supplemented with 5% FCS. MC57G (*H-2*^{b}) target cells, propagated in Dulbecco's modified MEM with 10% FCS, were infected with either recombinant vaccinia viruses (rVV) expressing the S 510-518 epitope (vJS510) or rVV expressing the *Escherichia coli lacZ* gene (vSC8) at a multiplicity of infection of 5 (2). Following incubation for 14 h at 37°C, target cells were labeled with 100 μCi of $Na^{51}CrO_4$ (New England Nuclear) for 1 h at 37°C. Target cells, at a concentration of 10^4 in a 100-μl volume, were added to the effector cells, and ^{51}Cr release was measured after 4 h of incubation. Data are expressed as percent specific release defined as [(experimental release) − (spontaneous release)]/[(total release − (spontaneous release)]. Maximum spontaneous release values were ≤20% of the total release value.

Serological tests. Serum neutralizing antibodies were assayed as previously described (5). Briefly, mice were sacrificed by CO_2 asphyxiation and blood was collected by cardiac puncture. Sera were heat inactivated at 56°C for 30 min prior to dilution. For assay, 50 μl of diluted serum and 100 μl of MEM containing 100 PFU of either JHMV or 2.2v-1 were added to 96-well microtiter plates. After incubation at 37°C for 1 h, 6×10^4 DBT cells in 50 μl were added to each well. Titers were recorded by visual inspection for cytopathic effect after 24 to 48 h of incubation at 37°C. The endpoint was defined as highest serum dilution at which no cytopathic effect was observed. Total anti-JHMV antibodies were determined by enzyme-linked immunosorbent assay (41).

Histology. Brains and spinal cords of three to four mice from each group were bisected in the mid-coronal plane and prepared for either paraffin or frozen sections. For paraffin preparation, tissues 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. Distribution of JHMV antigen was examined by immunoperoxidase staining (Vectastain-ABC kit; Vector Laboratory, Burlingame, Calif.), using the anti-JHMV MAb J3.3, specific for the carboxy terminus of the N protein, as the primary antibody (11) and horse anti-mouse MAb as the secondary antibody (Vector Laboratory). Frozen sections for labeling apoptotic cells were cut at 6 μm. An Oncor (Gaithersburg, Md.) ApopTag kit with terminal deoxynucleotidyltransferase (TdT) was used for end extension of fragmented DNA in apoptotic cells. Tissue processing and staining protocols were performed as indicated by the manufacturer.

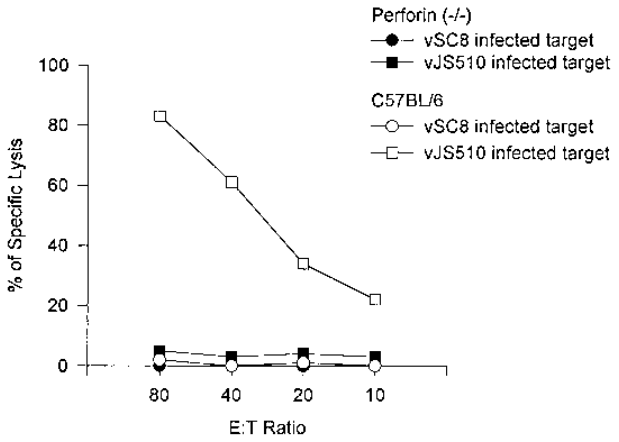

FIG. 1. T cells from perforin (−/−) mice are not cytotoxic. JHMV-immune splenocytes from perforin (−/−) or C57BL/6 mice were tested for cytolytic activity by using MC57G target cells infected with either rVV expressing the *E. coli lacZ* gene (vSC8) or the JHMV S 510-518 epitope (vJS510).

RESULTS

Role of perforin-mediated cytotoxicity in acute JHMV infection. To initially determine the extent of immunosuppression in perforin (−/−) mice and to ensure an absence of JHMV-specific perforin-mediated cytotoxicity, perforin (−/−) and C57BL/6 mice were immunized by intraperitoneal infection with 10^6 PFU of JHMV as previously described (2). Both groups of mice survived. Spleen cells from immunized perforin (−/−) mice stimulated with a peptide comprising the major *H-2*^{b}-restricted JHMV CTL epitope (S 510-518 epitope) were unable to lyse MC57G target cells infected with rVV expressing the S 510-518 epitope or a control rVV (vSC8) (Fig. 1). By contrast, spleen cells from JHMV-immunized C57BL/6 mice which were stimulated under identical conditions lysed target cells infected with the rVV expressing the S 510-518 epitope but not target cells infected with the vSC8 control virus. These data confirm the absence of perforin-mediated CTL activity in the perforin (−/−) mice (25, 47).

$CD8^+$ CTL, $CD4^+$ T cells, and NK cells are all recruited into the CNS during acute JHMV infection (10, 52); however, only $CD8^+$ and $CD4^+$ CTL have been directly implicated in reducing the infectious virus from the CNS (20, 44, 52). To investigate the role of perforin-mediated cytotoxicity in acute JHMV CNS infection, the clinical progression of disease and mortality of perforin (−/−) and (+/+) littermates and *H-2*-compatible C57BL/6 mice infected i.c. with JHMV were compared. Following a lethal JHMV infection, mice lacking the gene encoding perforin (−/−) showed slightly reduced clinical disease compared to both C57BL/6 mice and perforin (+/+) littermates (Fig. 2A). During the first 10 days postinfection (p.i.), the mortality rate (Fig. 2B) in perforin (−/−) mice was similar to that in the control groups, where individual mice succumbed to infection. In contrast to the rapid progression of disease in the controls, where 100% died within 10 days p.i., 20% of the perforin (−/−) mice showed prolonged survival until 16 days p.i. These data indicate that an absence of perforin-mediated cytotoxicity delays but does not eliminate virus-induced lethality. Typically, the perforin (−/−) mice devel-

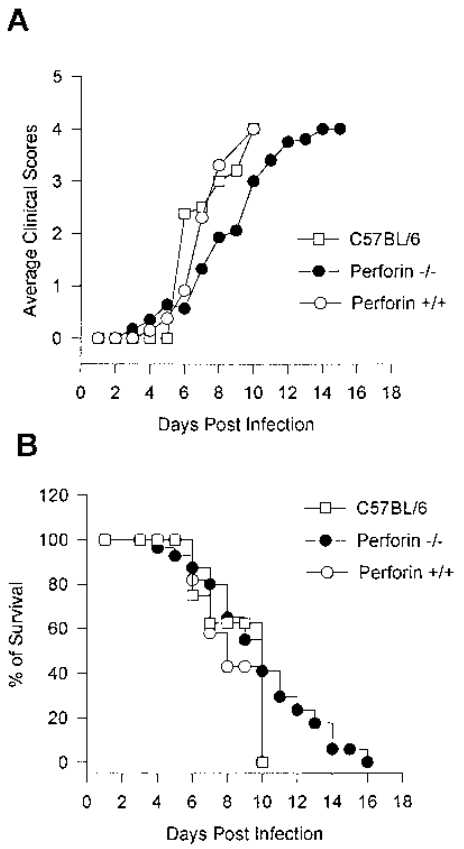

FIG. 2. Morbidity and mortality of perforin (−/−) mice undergoing acute JHMV infection. Groups of 12 to 16 C57BL/6, perforin (−/−), and perforin (+/+) mice were infected i.c. with 100 PFU of JHMV and observed for clinical scores (A) as described in Materials and Methods. (B) Survival of perforin (−/−), perforin (+/+), and C57BL/6 mice after lethal challenge infection with JHMV.

oped clinical disease at day 5 p.i. (ruffled fur) and slowly progressed to paralysis, wasting, and eventual death. The majority showed severe clinical disease (i.e., encephalitis and hind leg paralysis) for a protracted period compared to the control groups, consistent with increased survival without amelioration of disease. By contrast, both control groups developed early clinical signs of disease which progressed quickly to death within 24 h of onset. This is reflected by the rapid increases in clinical scores between 6 and 9 days p.i. (Fig. 2A). These observations suggest that $CD8^+$ CTL and possibly $CD4^+$ T cells directly contribute to the morbidity and mortality of CNS infection by JHMV.

Delayed clearance of JHMV from the CNS. These experiments suggested that the presence of perforin-mediated cytotoxicity correlated with more severe clinical disease. To determine whether the reduced severity of the disease in the perforin (−/−) mice was due to a reduced viral load or immunopathology, the kinetics of virus replication and extent of immunopathology were examined. The amount of infectious virus in the CNS of perforin (−/−) mice was equal to that in the controls at day 3 and then increased compared to the control groups at days 5, 7, and 10 p.i. (Fig. 3). Although the increase was not statistically significant at day 5 p.i., virus titers in the perforin (−/−) mice at day 7 p.i. were significantly higher than the titers in either perforin (+/+) or C57BL/6 controls. This trend continued to day 10 p.i., when 10^4 infectious virus was recovered from the perforin (−/−) mice, but no infectious virus could be recovered from the moribund control mice. These data confirm not only that perforin-mediated cytolysis is important for the clearance of JHMV from the CNS but also that it can also contribute to clinical disease. Although administration of neutralizing MAb also reduces viral replication (5), no neutralizing antibodies were detected in sera from perforin (−/−), perforin (+/+), or C57BL/6 mice at 7 days p.i. (data not shown). These data suggest that the effective clearance of JHMV from the CNS requires perforin-mediated lysis and that other mechanisms which may not involve neutralizing antibodies also participate in the clearance of JHMV from the CNS.

Virus-induced histopathological changes were examined to determine if the absence of perforin-mediated cytotoxicity and reduction in disease severity were due to changes in either the distribution of viral antigens or extent of encephalomyelitis. The amount of viral antigen correlated with the recovery of infectious virus from the CNS in both groups. Figure 4 shows representative sections from mice 10 days p.i. Viral antigen is present in glial cells of the perforin (−/−) mice (Fig. 4A), while little antigen could be detected in control mice (Fig. 4B) despite the presence of encephalomyelitis. The presence of encephalomyelitis in the perforin (−/−) mice confirms that trafficking of activated T cells into the CNS was not hindered in the absence of perforin-mediated cytolysis (25, 47). In addition, perforin (−/−) mice with increased viral antigen and encephalomyelitis had reduced clinical disease compared to control groups, suggesting a role for perforin-mediated cytotoxicity in clinical disease. The difference in clinical disease cannot be explained by an altered distribution of viral antigen due to perforin deficiency. While a few infected neurons were observed in both groups, glial cells were the predominant cell types infected with JHMV in both groups.

JHMV-specific CTL induce apoptosis in viral infected target cells in vitro (37). The extent of JHMV-induced apoptosis was examined in situ by the TdT labeling method to determine if the differences in disease severity correlated with the induction of apoptosis in vivo (Fig. 5). In both the perforin (−/−) mice and controls (Fig. 5A and B, respectively), numerous apoptotic cells were present at the sites of viral antigen and mononuclear cell infiltration. However, increased apoptosis, proportional to the increased viral antigen, was observed in the perforin (−/−) mice. To ensure that the apoptotic cells were related to the

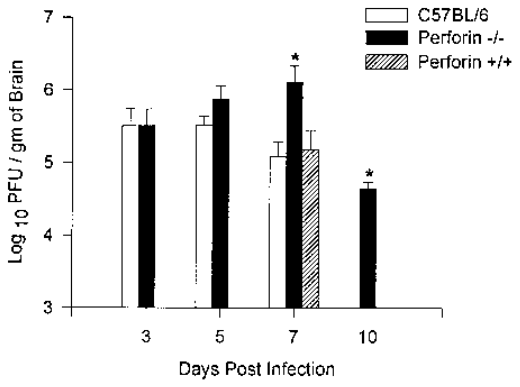

FIG. 3. JHMV titers in the brains of acutely infected perforin (−/−), perforin (+/+), and C57BL/6 mice. Titers were determined by plaque assay on confluent monolayers of DBT cells. Each time point is the mean of four to six samples, and the calculated standard errors are expressed as error bars. Student's *t* tests were used to test for significance between the two groups, and * denotes $P < 0.05$.

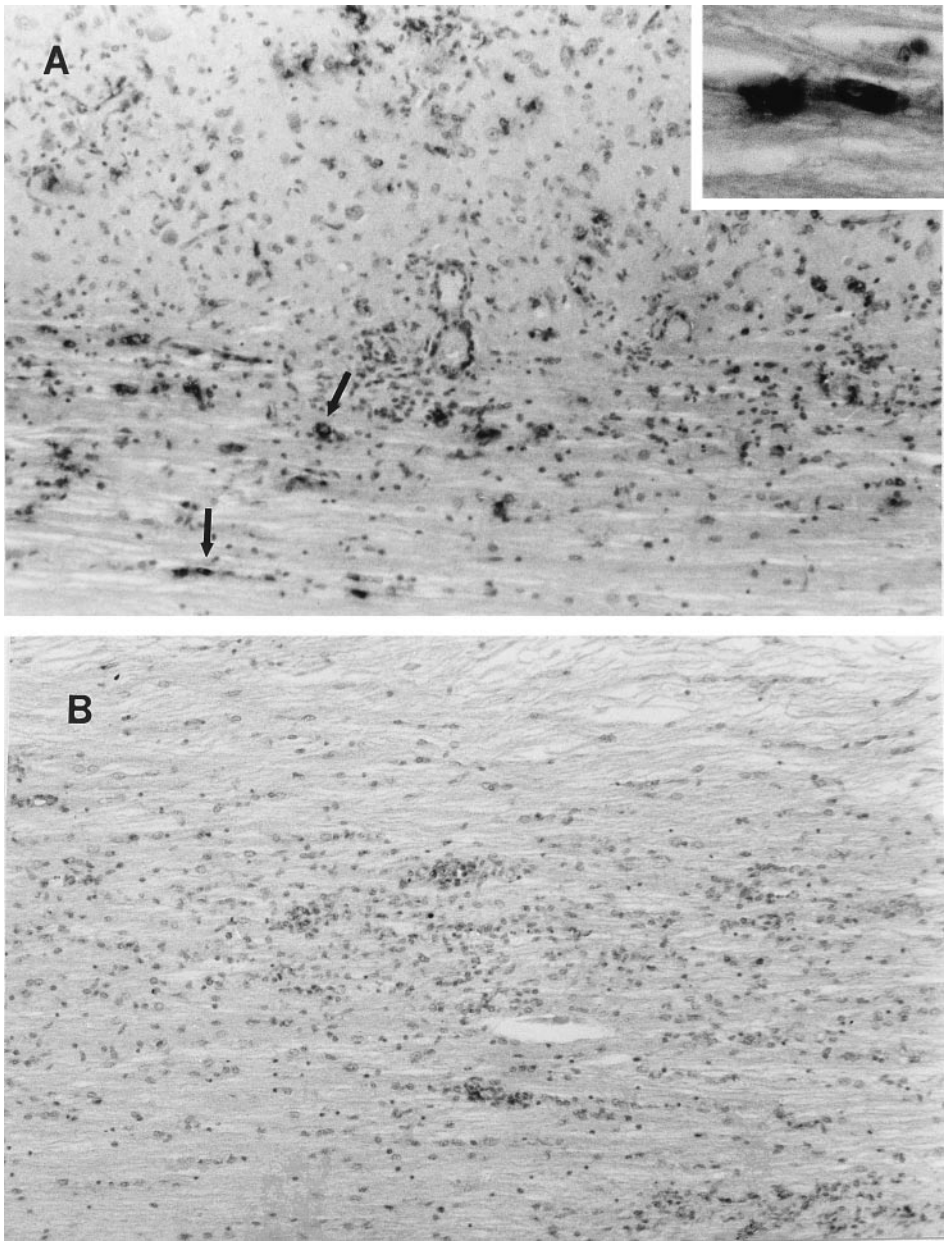

FIG. 4. Encephalomyelitis and JHMV antigen in infected perforin (−/−) and C57BL/6 mice. (A) Spinal cord tissues from perforin (−/−) mice 10 days p.i. show extensive cellular infiltrates and JHMV-positive cells (arrows). Magnification, ×115. The inset shows a higher magnification (×400) of JHMV-infected oligodendrocytes. (B) Tissues from C57BL/6 mice 10 days p.i. show cellular infiltrates but no cells immunoreactive for JHMV antigen. Sections were stained with anti-JHMV MAb J3.3 and counterstained with hematoxylin and eosin. Magnification, ×115.

immune response and not to viral infection (37), mice immunosuppressed by irradiation were examined. Immunosuppressed mice had an accelerated course of clinical disease. Irradiated mice infected with the DM strain developed lethargy and wobbly gate at day 3 p.i. and then quickly progressed to death by day 5. Few apoptotic cells were present despite widely disseminated viral antigen (data not shown). These data are consistent with the notion that a mechanism(s) other than perforin-mediated cytotoxicity induces apoptosis within the CNS during JHMV infection. Furthermore, JHMV infection in the absence of immune effectors is extremely lethal (49) and does not induce apoptosis in vivo.

Role of perforin in subacute infection. To determine if the absence of perforin-mediated cytotoxicity altered virus-induced demyelination, groups of perforin (−/−) and control mice were infected with a MAb-derived neutralization escape variant, 2.2v-1. This JHMV variant induces subacute encephalomyelitis followed by extensive demyelination (12). The acute clinical disease in the perforin (−/−) mice infected with 2.2v-1 was not different from the disease in the controls (Fig. 6). None of the mice showed signs of encephalitis until day 9. As infection progressed from 9 to 14 days p.i., all groups had similar courses of disease. Mice progressed from mild encephalitis (ruffled fur, hunched back, and minimum gait abnormal-

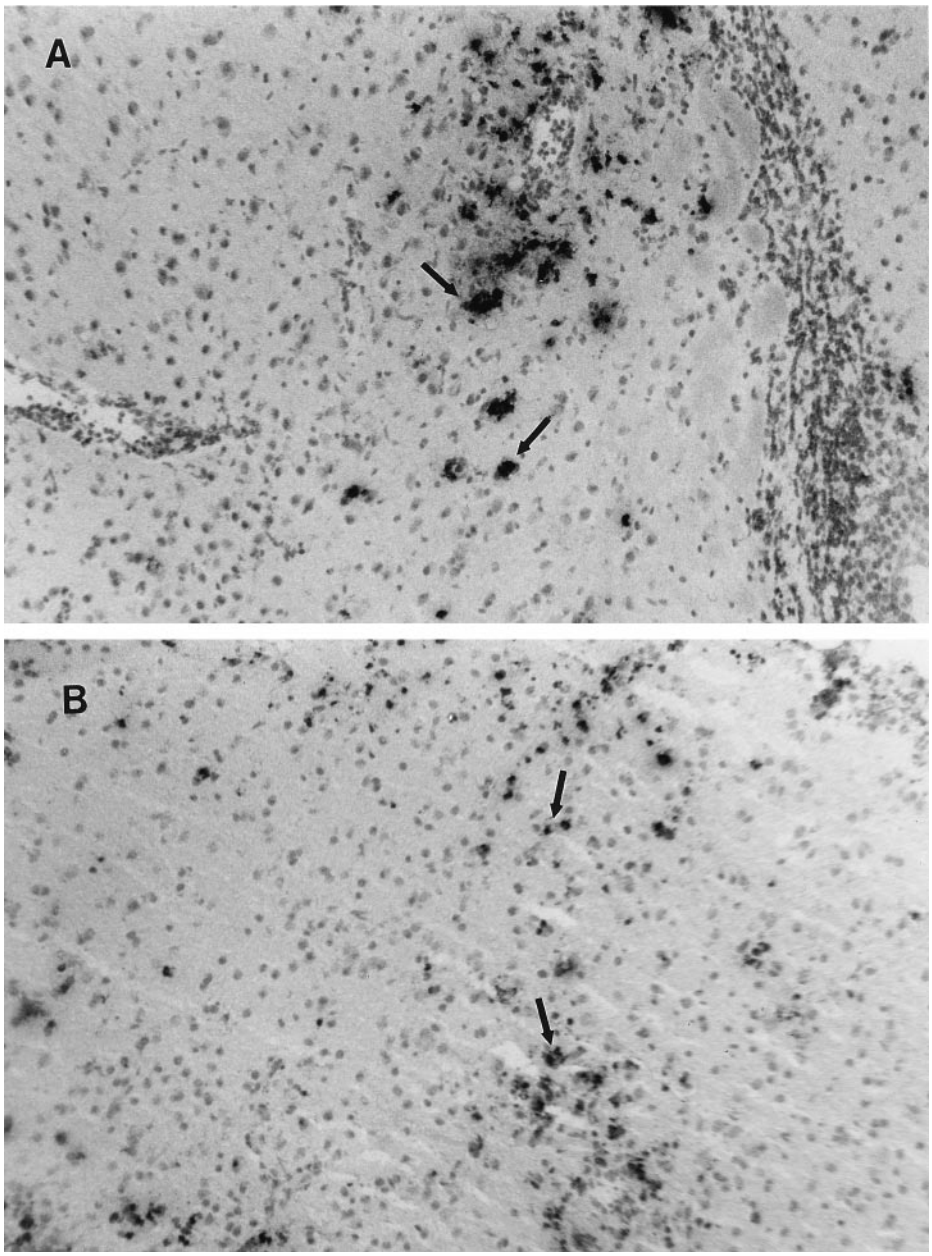

FIG. 5. Apoptotic cells (arrows) in brains of acutely infected perforin (−/−) and C57BL/6 mice. Cells undergoing apoptotic DNA fragmentation in the brains from day 5 p.i. perforin (−/−) (A) and C57BL/6 (B) mice were labeled by TdT. Magnification, ×115.

ity) to moderate paraparesis. Clinical improvement was evident by 15 days p.i. for all groups as expected (15), although the extent of improvement in the perforin (−/−) mice was less than that observed for the control groups. As the control mice proceeded toward clinical recovery between 15 and 30 days p.i., the perforin (−/−) mice remained lethargic and moderately immobile. At 30 days p.i., these mice had progressed to hind leg paralysis. No differences in the amount of viral antigen or cellular infiltrates were detected until 9 days p.i., when cellular infiltrates were present equally in both groups; however, decreasing viral antigen was found in the control group. At 30 days p.i., demyelination in the perforin (−/−) mice was extensive, confirming the clinical data (Fig. 7). This result suggests that perforin-mediated cytolysis is not the main effector of demyelination during JHMV infection. In both the control perforin (+/+) and perforin (−/−) mice, approximately equal amounts of viral antigens were still present in oligodendrocytes at 30 days p.i., suggesting that perforin-mediated cytotoxicity may not influence the development of persistent infection.

Reduced rate of viral clearance in perforin (−/−) mice. As for acute JHMV infection, reduced clearance of 2.2v-1 from the CNS of perforin (−/−) mice was found (Fig. 8). In perforin (−/−) mice, 2.2v-1 replication peaked at 3 days p.i. and then slowly declined until 14 days p.i., when virus was no longer detected in the CNS. Analysis of the kinetics of virus replication in the CNS of the control mice showed that infectious virus peaked at 3 days p.i., declined by 5 days p.i., and was below detectable levels by 8 days p.i. (12). Moreover, compar-

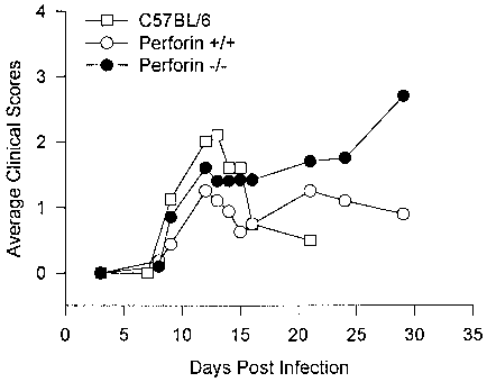

FIG. 6. Clinical course of 2.2v-1 infection in perforin (−/−), perforin (+/+), and C57BL/6 mice. Groups of 12 to 16 mice were infected i.c. with an antibody neutralization escape variant (2.2v-1) that results in a subacute demyelinating encephalomyelitis. Mice were scored for clinical disease as described in Materials and Methods.

ison of the kinetics of viral clearance between the acute and persistent infections (Fig. 8 and 3) shows that 2.2v-1 was cleared much more slowly than parental JHMV, consistent with previous results (12). Similar to the lethal JHMV infection, 2.2v-1 was eventually cleared from the CNS in the absence of perforin-mediated cytolysis (Fig. 8).

To assess the participation of an antiviral humoral response in the perforin (−/−) and controls infected with 2.2v-1, total serum immunoglobulin (Ig) and neutralizing antibodies were compared. No quantitative differences in total anti-JHMV Ig between the two groups were detected until day 14 p.i., when the total Ig level in the perforin (−/−) mice was approximately five times that in the control mice (data not shown). This result suggests that perforin (−/−) mice may compensate for a lack of perforin-mediated cytotoxicity by inducing a stronger humoral response or that increased antigen in the perforin (−/−) mice induces a stronger humoral response. Furthermore, the increase in total Ig corresponds with a similar increase of neutralizing activity in the perforin (−/−) mice. Figure 9 shows the kinetics of neutralizing antibody induction in these mice. No neutralizing antibodies were detectable prior to day 9 p.i. in either group, consistent with the data obtained during the acute infection. These data further support the absence of a correlation between the clearance of JHMV from the CNS and induction of the neutralizing antibody response.

DISCUSSION

This study was undertaken to identify the contribution of perforin-dependent cellular cytolysis in the pathogenesis of JHMV. Infection of perforin (−/−) mice with two different JHMV strains resulted in delayed viral clearance compared to control mice, confirming that perforin-dependent CTL participate in the elimination of JHMV from the CNS (44, 51). This delay was more apparent in perforin (−/−) mice infected with the 2.2v-1 variant, which is cleared more slowly from the CNS, possibly due to its predominant but not exclusive tropism for oligodendrocytes (12). Parental JHMV infects all glial cell types (12, 44, 45), and JHMV-specific CTL are more effective at reducing infection of astrocytes and microglia compared to oligodendroglia (44).

The immune response to JHMV infection of the CNS appears to involve a number of potential effector mechanisms critical for elimination of infectious virus from the CNS. These include $CD8^+$ CTL, $CD4^+$ T cells, and both virus neutralizing and nonneutralizing antibodies (28). Elimination of either the $CD4^+$ or $CD8^+$ T-cell subset prevents JHMV clearance from the CNS (51). $CD4^+$ T cells, however, do not appear to be a primary effector but provide help during CTL induction (41, 46, 51). This notion is consistent with the finding that adoptive transfer of JHMV-specific $CD4^+$ T cells protect recipients from lethal infection without reducing virus titer (26, 41, 46). The precise role(s) of $CD4^+$ T cells is not clear since some clonal JHMV-specific $CD4^+$ T cells mediate protection via partial reductions in virus replication (26, 53). These data

FIG. 7. Demyelination in perforin (−/−) mice infected with the 2.2v-1 variant of JHMV at 30 day p.i. Tangential sections through the white matter of the spinal cord were stained with luxol fast blue. Areas of demyelination are outlined by arrows. Magnification, ×115.

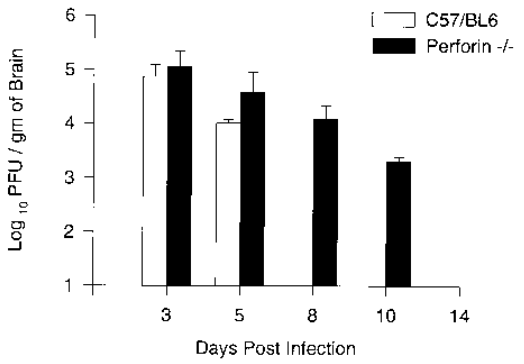

FIG. 8. Viral titers in perforin (−/−) and control mice following infection with the 2.2v-1 variant of JHMV. Titers were determined by plaque assay, and each time point represents the mean of four to six samples. Standard errors are expressed as error bars.

suggest possible roles for both $CD8^+$ and $CD4^+$ T-cell-mediated cytolysis in the pathogenesis of JHMV. NK cells, which also lyse targets via a perforin-dependent mechanism(s), rapidly traffic into the CNS in advance of both $CD8^+$ and $CD4^+$ T cells following JHMV infection (52). However, nude mice are unable to clear virus from the CNS (20, 36), consistent with the early increase in JHMV replication in the CNS of the perforin (−/−) mice.

An interesting finding was the eventual elimination of infectious virus from the CNS of perforin (−/−) mice infected with either JHMV strain. These data suggest that clearance of JHMV requires both perforin-dependent and perforin-independent antiviral responses. The nature of this additional antiviral response is unknown; however, both antibodies and antiviral cytokines have been implicated (5, 36). Neutralizing MAbs protect mice and reduce virus replication within the CNS, while nonneutralizing JHMV-specific MAbs are associated with protection in the absence of reduced viral replication (13, 30, 35, 54). Consistent with these data, nonneutralizing JHMV-specific antibodies were detected first on day 5 p.i. in all groups. By contrast, neutralizing antibodies were not detected until day 9 p.i., when viral replication within the CNS has already substantially decreased. These data support non-perforin-dependent cell-mediated cytotoxicity and antiviral lymphokines as likely candidates for the additional antiviral mechanism(s). The involvement of tumor necrosis factor (TNF) can be ruled out, as inhibition of TNF alters neither the influx of immune cells into the CNS nor virus replication during JHMV infection (45). The possibility that Fas-dependent cytolysis is associated with $CD4^+$ T cells which partially reduce virus replication in the CNS has not been addressed. However, although Fas-dependent cytotoxicity is induced during LCMV infection, it is insufficient to resolve the infection (25). Both in the transgenic model of hepatitis B virus (8, 19) and in mice infected with a hepatotropic strain of MHV (MHV-3), gamma interferon (IFN-γ) ameliorates disease and reduces viral replication (38). Furthermore, IFN-γ exerts an antiviral effect in the CNS during vaccinia virus replication (27). Whether IFN-γ or other potential antiviral cytokines, other than TNF, are involved in JHMV pathogenesis remain to be tested.

In this study, no reduction in mortality was found in perforin (−/−) mice undergoing a lethal JHMV infection compared to controls. These data differ from the analysis of perforin (−/−) mice undergoing LCMV-induced choriomeningitis, which resulted in reduced morbidity and no mortality (25). This difference may represent different tissue tropism or the absence of an exclusive CTL-mediated protective immune response. However, perforin deficiency was associated with reduced early morbidity following JHMV infection, supporting a role of perforin-mediated cytolysis in clinical disease. The possibility that reduced morbidity in the perforin (−/−) mice was due to the inability of CTL to induce apoptosis in virus-infected cells in vivo was excluded, as extensive numbers of apoptotic cells were found in both the perforin (−/−) mice and controls. Although the identity of these cells could not be confirmed due to ongoing apoptotic processes, it is likely that they represent a combination of both infiltrating mononuclear cells and virus-infected cells. The presence of antigen-positive cells undergoing apoptosis confirms in vitro data showing apoptosis associated with CTL-mediated cytolysis of virus-infected targets (37). Furthermore, few apoptotic cells were detected in immunosuppressed mice, supporting the in vitro finding that viral replication alone was unable to induce apoptosis (37).

A predominant pathological finding in JHMV infection is the presence of primary demyelination confined to the CNS. In contrast to their role in viral clearance, it is unclear whether CTL participate directly in the process of primary virus-induced demyelination. Cytolysis of virus-infected oligodendrocytes was initially reported to be associated with primary demyelination (29). The finding of CTL within the CNS of JHMV-infected mice with persistent demyelination (6) further supports this notion. By contrast, no demyelination was found in mice immunosuppressed by irradiation (14) or SCID mice (20); however, demyelination had been detected in JHMV-infected athymic nude (20) and β-2-microglobulin-deficient mice infected with the related MHV-A59 strain (17). Data in this report indicate that perforin-mediated cytolysis is not required for the demyelination process. Demyelination was neither prevented nor diminished following infection of perforin (−/−) mice with the 2.2v-1 JHMV variant. These mice had extensive demyelination, consistent with increased viral infection of oligodendrocytes (12) and the requirement for macrophages to mediate the process of demyelination (21, 48).

In summary, these data support the concept that JHMV is eliminated in vivo via both perforin-mediated cytotoxicity and a non-perforin-mediated immune response. Lytic CTL contribute to the clinical disease observed during acute infection and affect the process of demyelination by reducing the extent of viral infection. CTL appear to have little or no direct role in the primary demyelination associated with JHMV infection except as a mediator of virus elimination. Even though these experiments demonstrate the importance of perforin-mediated

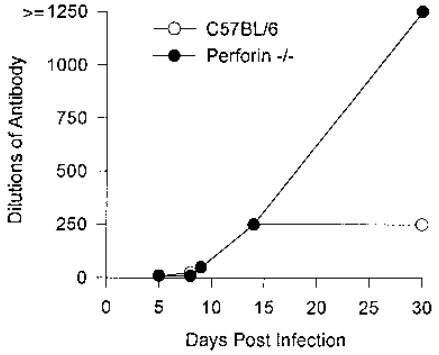

FIG. 9. Neutralizing antibody titers in perforin (−/−) and control mice infected with the 2.2v-1 variant of JHMV. Sera were collected from groups of three mice and tested for neutralizing antibodies as described in Materials and Methods. Results are expressed as the reciprocal of the dilution at which no cytopathic changes were observed.

cytotoxicity in the pathogenesis of JHMV CNS infection, they suggest an additional role of nonperforin-mediated immune mediators. Cytotoxicity mediated via the Fas/Fas ligand pathway and cytokines such as IFN-γ may also participate in the clearance of virus from the CNS, in particular the clearance of infectious virus from oligodendroglia.

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