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Hemagglutinin-Esterase-Specific Monoclonal Antibodies Alter the Neuropathogenicity of Mouse Hepatitis Virus

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Some of mouse hepatitis virus strains contain an optional envelope glycoprotein, hemagglutinin-esterase (HE) protein. To understand the functional significance of this protein, monoclonal antibodies (MAbs) specific for this protein were generated and used for passive immunization of mice. None of these MAbs showed any virus-neutralizing activity in vitro; however, mice passively immunized with the purified MAbs were protected from lethal infection by the JHM strain of mouse hepatitis virus. Passive immunization altered the pathogenicity such that the virus caused subacute and chronic demyelination instead of acute lethal encephalitis. Virus titers in the brains of the immunized mice were significantly lower than those for the nonimmunized control mice, suggesting that the virus replication or spread was inhibited. In addition, histopathological analysis indicated that the spread of virus in the brain and spinal cord was significantly inhibited in the immunized mice. Furthermore, the mononuclear cell infiltration in the immunized mice appeared earlier than in the nonimmunized mice, suggesting that the exogenous antibody might have activated host immune responses, and thus facilitated clearance of the virus or virus-infected cells. The same protective effects were observed for both JHM(2) and JHM(3) viruses, which expressed different amounts of the HE protein. In contrast, mice infected with At11f, a variant of JHM which does not express the HE protein, were not protected by these MAbs, suggesting that protection was mediated by the specific interaction between the MAb and the HE protein. Thus, the mechanism of protection by the exogenous HE-specific MAbs may represent the early activation of innate immune mechanisms in response to the interaction between the MAbs and the HE protein.

Mouse hepatitis virus (MHV) is a coronavirus, which contains three or four structural proteins: a spike (S) protein, a membrane (M) protein, a nucleocapsid (N) protein and, in some strains, a hemagglutinin-esterase (HE) protein. S protein is an envelope glycoprotein responsible for receptor binding on host cells and elicits neutralizing antibodies (4). M protein is a transmembrane glycoprotein (21), and N protein is a phosphorylated protein which interacts with viral RNA (34). HE is an envelope glycoprotein present in certain species of coronavirus, e.g., bovine coronavirus (BCV) (15, 26, 35), turkey coronavirus (5), human coronavirus strain OC43 (13, 14), and some strains of MHV (30, 36, 37). In contrast, no HE or equivalent protein is present in avian infectious bronchitis virus (33), some strains of MHV (30), or porcine transmissible gastroenteritis virus (9).

Although the function of the HE protein is not clear, it is known that its expression in the JHM strain of MHV correlates with the copy number of an UCUAA pentanucleotide at the 3′ end of the leader sequence (30, 36), located at the 5′ end of the viral genome. This repetitive sequence is homologous to the intergenic transcriptional initiation signals preceding each gene on the viral genome, and its repeat number varies from two to four, depending on the virus isolate (22). There is a tendency for the copy number in the viral genome to decrease from three to two during viral passage in tissue culture cells at a high multiplicity of infection (22). We have previously isolated, from the same virus stock, two viruses which possess either two or three copies of the UCUAA repeat, designated JHM(2) and JHM(3), respectively (30, 37). JHM(3) expressed a small amount of the HE protein, whereas JHM(2) expressed at least 10 times more HE protein in both infected cells and the mature virions (30, 35a, 37). Interestingly, when injected intracerebrally into mice, these viruses showed different pathogenicities in terms of both virulence and cellular tropism (35a). JHM(2)-infected mice had a mean time of death at day 7 postinfection (p.i.), while JHM(3)-infected mice survived an average of 14 days. Furthermore, JHM(2) infected primarily neuronal cells in the gray matter, while JHM(3) infected predominantly glial cells in the white matter. Although these differences could not be ascribed unequivocally to the difference in the amount of HE protein in these viruses, these data suggest that the HE protein might possibly be involved in viral pathogenesis.

As an approach to evaluating the possible function of the MHV proteins in vivo, passive immunization experiments have been performed with monoclonal antibodies (MAbs) specific for S, M, and N proteins (3, 7, 25). Passive immunization of JHM-infected mice with MAbs specific for either the S or M protein altered the viral neuropathogenicity from acute lethal encephalitis to subacute or chronic demyelination (3, 7). Passive immunization with MAbs specific for the N protein also changed the pathogenicity of MHV-2 infection by inducing a prominent cellular infiltration and protected mice from lethal hepatitis (25). The mechanism of passive protection by these MAbs, especially by the nonneutralizing MAbs, is not clear. However, in the case of JHM infection of the central nervous system, the sparing of neurons from viral infection in protected mice has been a consistent finding.

In the present study, MAbs specific for the HE protein of JHM were generated. None of them showed virus neutralization activity in vitro; however, passive immunization by

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all MAbs protected JHM-infected mice from a lethal acute encephalomyelitis and resulted instead in subacute and subsequently chronic demyelination. The mechanism of passive protection was investigated.

MATERIALS AND METHODS

Viruses and cells. JHM(2), which contains two UCUAA copies at the 3′ end of the leader sequence, and JHM(3), which contains three UCUAA copies (22, 30), were obtained from the original JHM stock after 14 passages at a multiplicity of infection of 2 to 5 in DBT cells, a murine astrocytoma cell line (12). These two virus isolates were plaque purified three more times before being used in this study. A JHM variant, At11f(brain), derived from the brain of an infected Wistar Furth rat (24), was a gift from V. Morris, University of Western Ontario, London, Ontario, Canada. This virus contains a 739-nucleotide deletion in the HE gene (18), resulting in the complete loss of detectable HE protein expression. At11f was passaged in DBT cells five times and plaque purified twice. The plaque isolates were further passaged twice in DBT cells before being used. Virus isolates of At11f were examined for their mRNA structures by analysis of ^{32}P-labeled intracellular virus-specific RNAs (36) and/or by polymerase chain reaction (36) to confirm the original phenotype; i.e., the HE gene was partially deleted (18).

Animals. Six-week-old male C57BL/6 mice were obtained from Jackson Laboratories, Bar Harbor, Maine. The animals were inoculated intracerebrally (i.c.) in the left hemisphere with the indicated viruses in a total volume of 32 μl.

Isolation of hybridomas producing HE-specific MAbs. C57BL/6 mice were immunized with the clarified supernatant from JHM(2)-infected DBT cells. Mice were injected intraperitoneally (i.p.) five times during a year, with two injections with Freund's complete adjuvant and the remaining three injections with incomplete adjuvant (Sigma). The final immunization was done by using virus which had been irradiated with UV (UV-254nm Mineralight lamp; ULTRAVIOLET Products Inc. San Gabriel, Calif.) for 5 min at 4°C, without any adjuvant. Hybridoma cells were generated by polyethylene glycol-mediated fusion by using myeloma cell line HL-1 (Ventrex, Portland, Maine), a subclone of NS-1 cells derived from BALB/c mice, by modification of the procedures published previously (17). Hybridoma-positive wells were initially screened for anti-MHV antibody by enzyme-linked immunosorbent assay (ELISA), with clarified supernatant from the JHM(2)-infected DBT cells as antigen (8). Antigen specificity was determined by Western blot (immunoblot) and confirmed by radioimmunoprecipitation. Western blots were performed with JHM(2)-infected DBT cell lysates. Briefly, cell lysates were electrophoresed on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) and transferred to a nitrocellulose membrane (Immobilon NC; Millipore, Bedford, Mass.). Antigens were visualized by using 4-chloronaphthol as a substrate according to the procedure recommended by the manufacturer (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif.). Immunoprecipitations were performed with [^{35}S]methionine-labeled JHM(2)-infected cell lysates as previously described (37). Precipitates were analyzed by SDS–12.5% polyacrylamide gel electrophoresis (PAGE) (37). Positive clones were subcloned by limiting dilution. Immunoglobulin (Ig) subclass was determined by double immunodiffusion (Outerlony technique; agarose tablet from Bio-Rad Laboratories, Richmond, Calif.) with rabbit anti-Ig subclass antibodies (Cappel Organon Teknika, Durham, N.C.).

Purification of IgG. Serum-free medium from the hybridoma cultures was clarified by low-speed centrifugation and applied to a protein A/G affinity column as recommended by the manufacturer (Pierce, Rockford, Ill.). After elution, the peak fractions at an optical density of 280 nm were combined and dialyzed extensively against phosphate-buffered saline (PBS), pH 7.2. Protein concentration was determined by Bio-Rad protein assay, with bovine serum albumin (Promega) as a standard.

Virus neutralization assay. Approximately 10^3 PFU of JHM(2) virus was incubated with serial 10-fold dilutions of the purified MAbs (containing 5 ng to 50 μg of protein) in 1 ml of minimum essential medium at 37°C for 45 min. Virus neutralization was determined by plaque assay with DBT cell monolayers as previously described (8). Controls included JHM(2) incubated without antibody and the A59 strain of MHV, which did not express the HE protein (20, 36), incubated with the purified MAbs. As further controls, JHM(2) and A59 were also incubated with either a rabbit polyclonal antibody (37) or an MAb specific for the S protein (J.7.1) (8).

Esterase-inhibition assay. JHM(2) virus was precipitated from 100 ml of culture medium with 50% saturation of ammonium sulfate as described previously (23) and resuspended in 2.0 ml of PBS. Ten microliters of virus suspension was incubated with serial 10-fold dilutions, ranging from 1.0 ng to 1.0 μg, of purified MAbs for 30 min and then mixed with *p*-nitrophenylacetate as substrate for esterase (35). Hydrolysis of the substrate was monitored at 400 nm at 2-min intervals as described previously (37).

Passive immunization. Mice were inoculated i.c. in the left hemisphere to a depth of approximately 3.0 mm, with 100 PFU of each virus in 0.032 ml of PBS. Purified IgG (100 μg in 0.2 ml of PBS) was immediately injected i.p. For determination of viral virulence, groups of infected mice (five to seven mice per group), which had received either IgG or PBS, were observed for mortality for up to 92 days p.i. The purified IgG1 (100 μg/0.2 ml of PBS) of a β-galactosidase-specific MAb obtained from a BALB/c mouse-derived hybridoma (Boehringer Mannheim) was inoculated i.p. as a control. For time course studies of histopathology and viral titer, three mice were sacrificed on each of days 3, 5, 7, 10, 13, 17, 20, and 100 p.i. One half of the brain from each mouse was used for histopathological analysis. The remaining half was homogenized in 2.0 ml of ice-cold Dulbecco's PBS to determine virus titers. Briefly, after clarification by low-speed centrifugation at 4°C, the supernatant was serially diluted 10-fold, and 0.2 ml of each dilution was used to infect DBT cells in 24-well plates, in quadruplicate. The sensitivity of this assay had a lower limit of approximately 20 PFU/g of tissue.

Histopathology. Brain, spinal cord, and spleen were immersion fixed for 3 h in Clarke's solution (75% absolute alcohol–25% glacial acetic acid) and embedded in paraffin. Sections (6 μm in thickness) were stained with hematoxylin-eosin. Brain and spinal cord were also stained with luxol fast blue and counterstained with eosin. For brain samples, two coronal sections at the levels of the pituitary gland and occipital pole, to include the posterior edge of the hippocampal fissure, were prepared. For spinal cord samples, one longitudinal section of the entire cord was prepared. For viral antigen detection, immunoperoxidase staining (Vectastain-ABC kit) was performed, as previously described (7), by using the N protein-specific MAb J.3.3 (8).

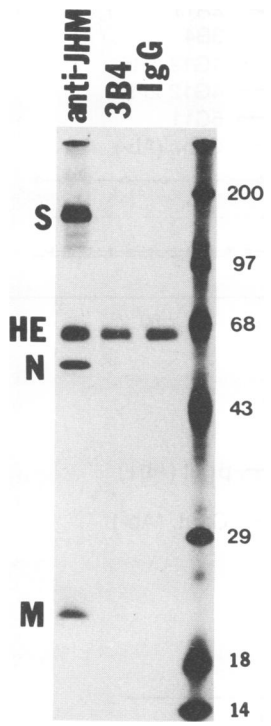

FIG. 1. Immunoprecipitation of JHM(2)-infected cell lysate with MAbs. [^{35}S]methionine-labeled lysates from MHV-infected cells were precipitated with purified IgG of one of the MAbs, 3B4, which was used for most of the experiments. Lanes labeled 3B4 and IgG represent two different preparations of IgG. The control lane (anti-JHM) was the same lysate precipitated with the polyclonal antibody against the purified JHM(2) virus (37). Molecular weight markers (in thousands) are indicated on the right.

RESULTS

Characterization of MAbs specific for HE. The supernatant fluids from fused myeloma cells were initially screened by ELISA, with the supernatant of the JHM(2)-infected DBT cells as antigen. Supernatants of the positive clones were then assayed by Western blot for their reactivities to the JHM viral proteins. Five clones which showed specificity for the HE protein were subcloned by limiting dilution and expanded, and the IgG fraction was purified from the hybridoma supernatants. Figure 1 shows immunoprecipitation of [^{35}S]methionine-labeled proteins from JHM(2)-infected cells with IgG of one (designated 3B4) of the HE-specific clones. Only the HE protein was precipitated (Fig. 1). Similar results were obtained with the other four clones (designated 2A11, 4G12-1H5, 4G12-2F9, and 5C11) (data not shown). The MAbs from these clones were tested for virus neutralization, suppression of virus-induced cell-to-cell fusion, and inhibition of esterase activity. None of the MAbs showed any of these activities. They were also examined for IgG subclass. MAb from one clone (4G12-2F9) was the IgG1 isotype, while the remaining four were IgG2A. The in vitro translation product of a truncated HE gene which contained the N-terminal 140 amino acids was precipitated by all five MAbs, suggesting that they recognized an epitope(s) within this domain. Preliminary competition assays suggested that all five MAbs may recognize the same or an overlapping epitope (data not shown).

Passive immunization of JHM(2)-infected mice. To study the possible role of HE protein in the pathogenesis of MHV, the effect of passive immunization with the HE-specific MAbs on JHM(2) infection of mice was first tested. Inoculation of JHM(2) virus, which synthesizes a large amount of HE protein (36, 37), i.c. into 6-week-old C57BL/6 mice at 100 PFU per mouse was immediately followed by i.p. injection of 100 μg of each purified MAb. Control mice, infected with the same virus, received an equal volume of PBS. Each group consisted of five mice except for the control group, which consisted of seven mice. Clinical signs were observed every other day until 92 days p.i. (Fig. 2A). All control mice developed hunch backs and rough fur on day 5 and died between 6 and 8 days p.i.

In contrast, most of the mice which had received HE-specific MAbs were free of gross clinical signs until approximately day 16 p.i., when all surviving mice began to exhibit hind-leg paralysis. This clinical sign typical of demyelination never cleared completely and was still observed in survivors on day 92 p.i. There was no difference in protective effect between MAbs of different IgG subclasses, including IgG1 (4G12-2F9) and IgG2A. A β-galactosidase-specific antibody (purified IgG1) was used to rule out nonspecific protection. As shown in Fig. 2B, immunization with 100 μg of β-galactosidase-specific antibody did not have any protective effect against JHM(2) infection, suggesting that the protective effect of HE-specific MAbs was specific.

Some of the surviving mice (40% of all mice) showed delayed clinical signs of encephalitis, similar to the delayed encephalitis observed for mice partially protected by the adoptive transfer of T cells from an immunized donor in a previous study (31), and died between 20 and 30 days p.i. Another peak of death (20% of all mice) was observed between 40 and 50 days p.i., when mice developed severe paralysis. These two clustered temporal windows of death were reproducible in three independent experiments.

These results showed that, even without in vitro virus-neutralization or esterase-inhibition activities, the HE-specific MAbs protected mice from lethal acute encephalitis caused by JHM(2) infection and altered the outcome of infection into a subacute demyelinating process.

The time course study of virus titers in the brain. To study the kinetics of virus growth in the passively immunized mice, virus titers in the brains of JHM(2)-infected mice immunized with the antibody 3B4 were determined, in the presence or absence of passive immunization (designated Ab+ and Ab−, respectively), by plaque assays (Fig. 3). Three mice from each group were sacrificed on the days indicated, and virus titers were averaged. The Ab− group on day 10 represented only a single mouse which showed acute encephalitic signs on day 6 but survived until day 10. The data for the Ab+ group were combined from two sets of experiments: one from days 0 to 10 and the other from days 7 to 20. The titers on days 7 and 10 in these two experiments were similar; therefore, the data were combined for presentation. Figure 3 shows that, during the acute phase of infection (up to 7 days p.i.), the virus titers in the Ab+ group were more than 1 $\log_{10}$ lower than those of the Ab− group. In particular, the virus titer in the Ab+ group on day 7 was 2 $\log_{10}$ lower than that of the Ab− group. This result suggested that, in the passively immunized mice, virus replication was partially inhibited. After day 10, the virus titers in the brains of the Ab+ group increased to the same level as those in the acute phase, suggesting the relapse of virus replication.

The time course study of histopathology. To study the localization of viral antigen in the brain and spinal cord,

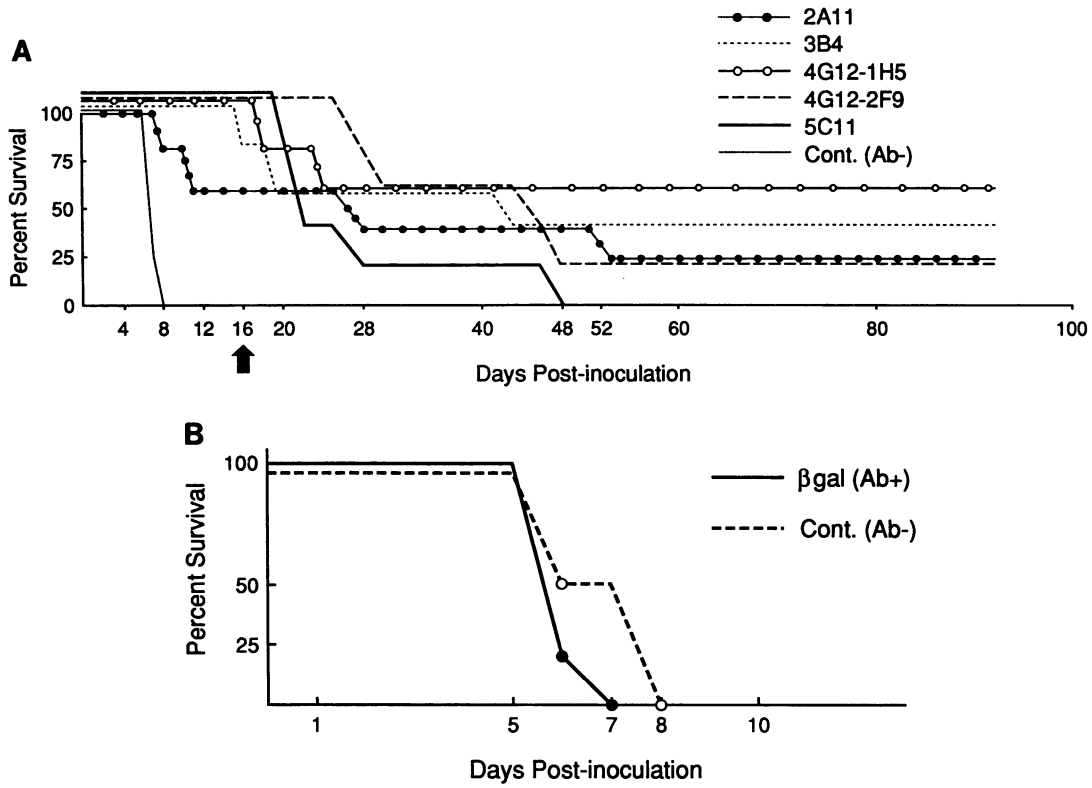

FIG. 2. Passive protection with MAbs of the mice against JHM(2) infection. Mice were infected i.c. with 100 PFU of JHM(2) virus and immediately inoculated i.p. with either 100 μg of purified IgG of different MAbs specific for its protein (A) or β-galactosidase (βgal)-specific MAb or PBS (control) (B). Mice were then observed daily for mortality. The percentages of surviving mice on each day are indicated. For panel A, each group consisted of five mice except for the control (Ab−) group, which had seven; for panel B, each group consisted of six mice. The arrow indicates the appearance of hind-leg paralysis in surviving mice.

immunoenzyme staining by using an MAb specific for the N protein was performed for both the Ab+ and Ab− groups. Figure 4 shows the comparison of the acute pathology between the Ab+ and Ab− groups. In the Ab+ group, viral antigen was restricted to ependymal cells on day 3 p.i. (Fig. 4a), whereas in the Ab− group, antigen was detected not only in ependymal cells and their neighboring cells (Fig. 4b) but also in neurons in the cortex (Fig. 4c). However, by day 5 p.i., the antigen distribution in the Ab+ group became similar to that in the Ab− group on day 3 p.i. In addition, in the Ab+ group, the mononuclear cell infiltration was prominent around cells containing viral antigen and in the perivascular areas, and viral antigen appeared to be localized in cell clusters (Fig. 4d). In contrast, no obvious mononuclear infiltration was observed in the Ab− group (Fig. 4e). In the spinal cord, viral antigen became detectable at about the same time (day 5 p.i.) for both groups; however, antigen was present mostly in ependymal cells or perivascular areas of the Ab+ group (Fig. 5a), whereas it was detected throughout the spinal cords of the Ab− group (Fig. 5b).

On day 7 p.i. for the Ab− group, viral antigen was localized predominantly in glial cells and infiltrating mononuclear cells became abundant. The majority of antigen was detected in the spinal cord, in which numerous antigen-positive glial cells and a few antigen-positive neurons were detected; there was mononuclear cell infiltration in areas of demyelination (data not shown). In contrast, for the Ab+ group, viral antigen was detectable in only a few cells in the brains and spinal cords, which were surrounded by mononuclear cells (data not shown).

On day 10 p.i., when viral titer began to increase again in the Ab+ group (Fig. 3), abundant viral antigen was detected in the white matter of the spinal cord accompanied by severe cellular infiltration and a small amount of demyelination (Fig. 6a and b). On day 13 p.i., the recurrence of viral infection was evident by increased viral titer and the presence of viral antigen in neurons and glial cells in both the brain and spinal cord (data not shown). On day 17 and thereafter, most of the viral antigen was restricted in glial cells in the spinal cord, especially those proximal to the cervix, while very little was detected in the brain. The apparent progression of viral antigen-positive cells from the brain through the brain stem to the spinal cord from day 13 to day 17 suggested the neural route of virus spread. By day 20, marked demyelination was noted in the spinal cords of some mice (Fig. 6c). The paucity of viral antigen in the brain contrasted with the detection of abundant infectious virus in the brain from day 13 to day 20 (Fig. 3). This discrepancy was probably due to the localization of virus in the portion of the spinal cord adjacent to the brain stem, which was routinely included in the preparation of brain homogenate for the virus titer assay.

Surviving mice never recovered from paralysis, even on day 100 p.i. For all the mice sacrificed on day 100 p.i., viral antigen was relatively abundant in the brains and spinal cords, as demonstrated by immunoenzyme staining with

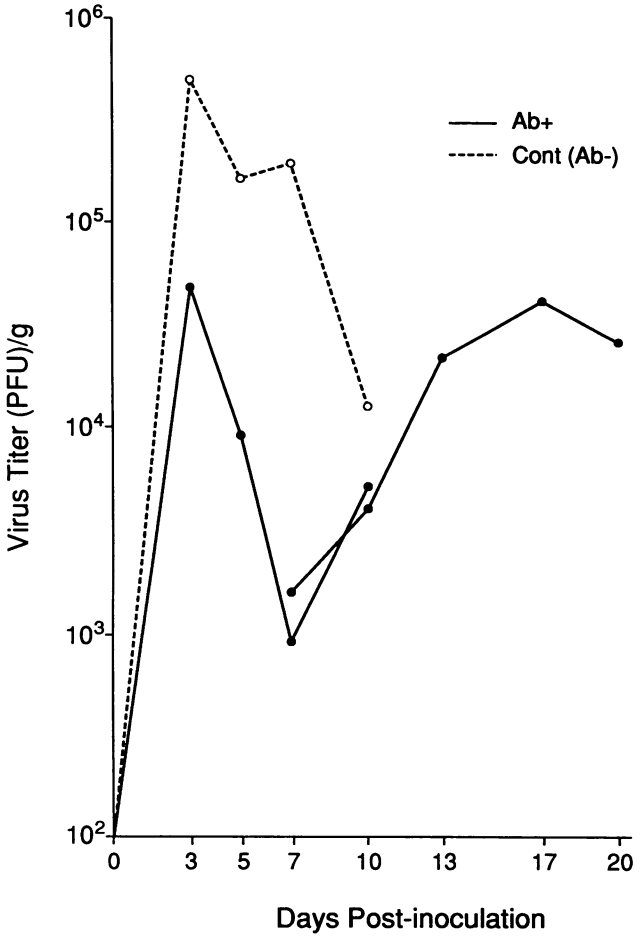

FIG. 3. The time course study of virus titer in the brain. Three mice each from the immunized and control mice were sacrificed on the indicated days after viral infection. The averages of virus titers in the brains of the three mice are presented as PFU per gram of brain tissue. The data for days 3 to 10 and 7 to 20 for the Ab+ group from two separate experiments were combined. The datum point on day 10 of the control (Ab−) group was obtained from a single surviving mouse. No mice in the Ab− group survived after day 10.

MAbs specific for either N protein (Fig. 7a and b) or S protein (data not shown). There were also areas of demyelination with signs of mononuclear cellular infiltration. The presence of viral RNA was detected by the polymerase chain reaction, which amplified the sequences in the S gene region of viral RNA (data not shown). However, no infectious virus could be isolated, suggesting that the virus in the brain might be defective.

Passive immunization of JHM(3)-infected mice. If the protective effect of the HE-specific MAbs was due to their interactions with the HE protein, one might predict that these MAbs would have a minimal effect on JHM(3) virus infection, since this virus synthesizes approximately 10-fold less HE protein than JHM(2) virus (35a, 37). To test this possibility, the protective effect of MAb 3B4 on JHM(3) infection was examined (Fig. 8). Control mice infected with 100 PFU of JHM(3) developed clinical signs of encephalitis and died between 10 and 14 days p.i., approximately 5 to 6 days slower than JHM(2)-infected mice (Fig. 8). In contrast, all of the JHM(3)-infected mice which had been passively immunized with MAb 3B4 survived until day 45. Similarly to the JHM(2)-infected mice which had been passively immunized, these mice began to show signs of paralysis on day 16 p.i. Although no mice died during the first window of mortality observed with JHM(2)-infected mice (between 20 and 30 days p.i.), two mice died in the second window, between 40 and 50 days p.i. Thus, in contrast to the prediction, the protective effects of HE-specific MAbs were similar for both JHM(2) and JHM(3) viruses. This finding suggested that the protective effects of HE-specific MAbs were unrelated to the quantity of HE proteins synthesized by the virus.

Passive immunization of mice infected with the HE-deletion mutant At11f. The finding that the HE-specific MAb altered the subacute encephalitis caused by JHM(3), which synthesized only a small amount of HE protein, similar to its effect on JHM(2) infection, raised a possibility that the protection may not be due to the specific interaction between the antibody and its ligand. To test this possibility, the effects of passive immunization on the infection by a JHM variant, At11f, which has a 739-nucleotide deletion in the HE gene, resulting in the total absence of the HE protein expression (18), were examined. Preliminary studies determined that 100 PFU of At11f virus was sufficient to cause death for all of the infected mice within 1 week, similar to the pathogenicity of JHM(2). C57BL/6 mice were then infected with 100 PFU of At11f virus, followed immediately by i.p. inoculation with the HE-specific MAb 3B4. Figure 9 shows that the clinical course of infection by this HE protein-deficient JHM variant was not altered by passive immunization with the HE-specific antibody, indicating that the protective effect of passive immunization with HE-specific antibody requires HE protein expression.

DISCUSSION

In this study, HE protein-specific MAbs were generated and characterized. Passive immunization with these MAbs protected mice from JHM-induced lethal acute encephalitis and resulted in subacute and chronic demyelination. Mice infected with JHM(3), which synthesized only a small amount of HE protein, were protected as efficiently as were mice infected with JHM(2), suggesting that protection was not dependent on the quantity of the expressed protein. However, we showed that the protective effects were mediated through the specific interaction between the HE protein and the MAbs since the mice infected with an HE protein-deficient JHM virus, At11f, were not protected; nor were mice protected by passive immunization with an irrelevant MAb. These findings raise interesting issues concerning the role of the HE protein in viral pathogenesis and replication.

With BCV, the HE protein has both hemagglutination and esterase activities and elicits neutralizing antibodies, suggesting that HE protein is required for BCV replication (6, 35). With MHV, the HE protein shows esterase and hemadsorption activities similar to those of BCV but only a very weak hemagglutination activity (27, 37). None of the MAbs tested in this study exhibits virus-neutralizing activity. We cannot, however, at the present time, rule out the possibility that some neutralization epitopes exist on the HE protein of MHV. The fact that some MHV strains do not synthesize this protein suggests that the HE protein is not necessary for MHV replication (36). Despite some sequence homology between MHV and BCV, particularly around the active esterase domain, the HE protein-specific MAbs to BCV did not cross-react with the MHV HE protein by immunopre-

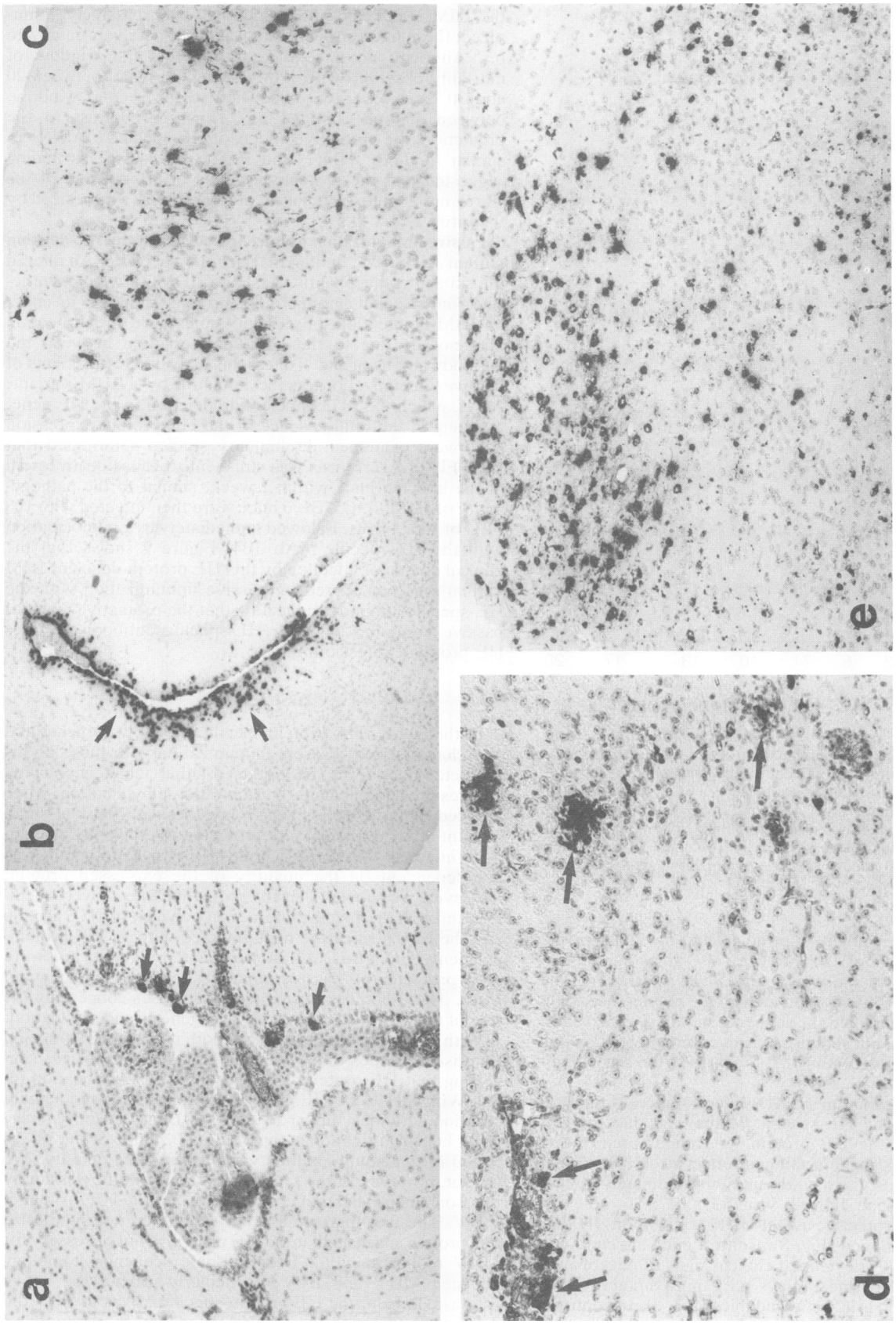

FIG. 4. Histopathology of JHM(2)-infected mouse brain in the acute stage of infection. The mouse brains from immunized and control groups were used for histopathological analysis every 2 to 3 days after infection. One section of brain was subjected to hematoxylin-eosin staining and another to immunoenzyme staining by using the N protein-specific MAb J.3.3 (8) as the primary antibody. Viral antigen distributions in the acute phase (days 3 and 5) were compared for nonimmunized (Ab−) and immunized (Ab+) groups. Representative cells containing viral antigen are indicated by arrows. (a) For the Ab+ group on day 3 p.i., area surrounding the lateral ventricle, showing ependymal cells positive for viral antigen. Magnification, ×350. (b) For the Ab− group on day 3 p.i., the same area as shown in panel a. Magnification, ×140. (c) For the Ab− group on day 3 p.i., cerebral cortex, showing the predominance of viral antigens in neurons. Magnification, ×350. (d) For the Ab+ group on day 5 p.i., cerebral cortex, showing perivascular cell infiltration and aggregations of cells positive for viral antigen. Magnification, ×700. (e) For the Ab− group on day 5 p.i., cerebral cortex. Viral antigen is predominantly in neurons and some glial cells. Magnification, ×350.

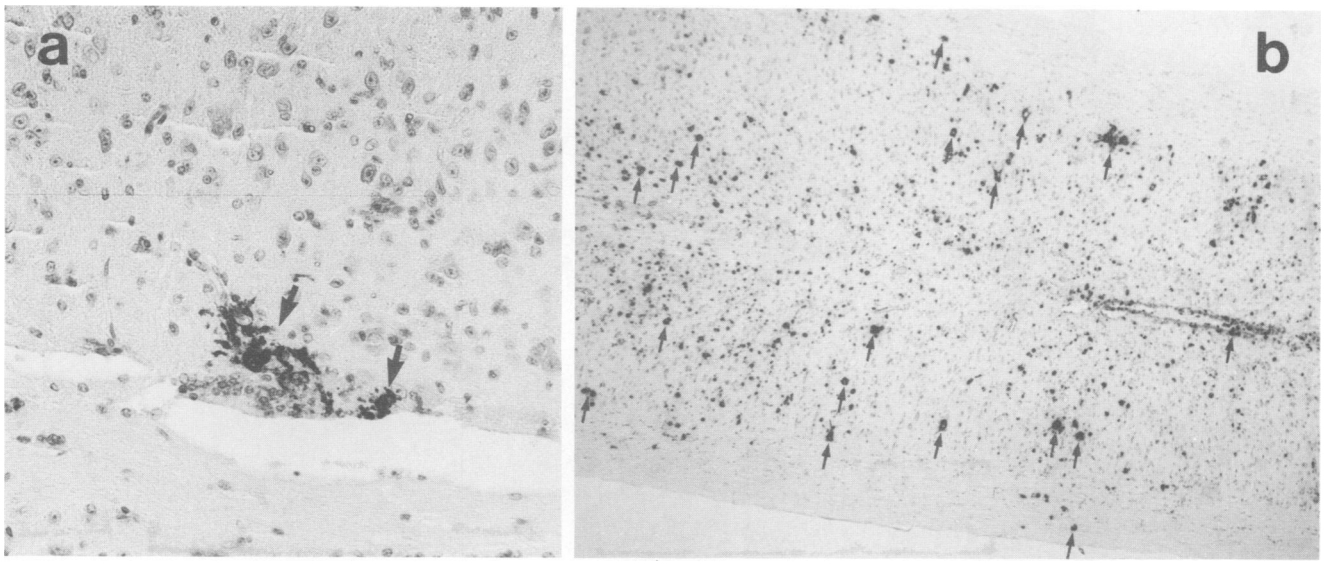

FIG. 5. Immunoenzyme staining of viral antigen in the spinal cord on day 5 p.i. Longitudinal sections of spinal cord from three infected mice each of immunized and control groups were analyzed. Representative cells containing viral antigen are indicated by arrows. (a) Ab+ group on day 5 p.i. Viral antigen was limited in the area surrounding the central canal. Magnification, ×1,400. (b) Ab− group on day 5 p.i. Viral antigen was found in glial cells throughout the spinal cord. Magnification, ×140.

cipitation (unpublished observation), suggesting that the HE proteins of BCV and MHV may be functionally distinct and have different roles in viral replication.

What is the mechanism of protection by the HE protein-specific antibodies? Protective effects have been demonstrated with nonneutralizing MAbs specific for S, M, and N proteins of MHV (3, 7, 25). It has been postulated that complement-mediated cytolysis or antibody-dependent cytotoxicity are responsible for the protection by nonneutralizing MAbs in various virus systems (7, 10, 19, 25, 28, 29, 32). It has also been suggested that protection by nonneutralizing MAbs may be due to alteration of cellular tropism of

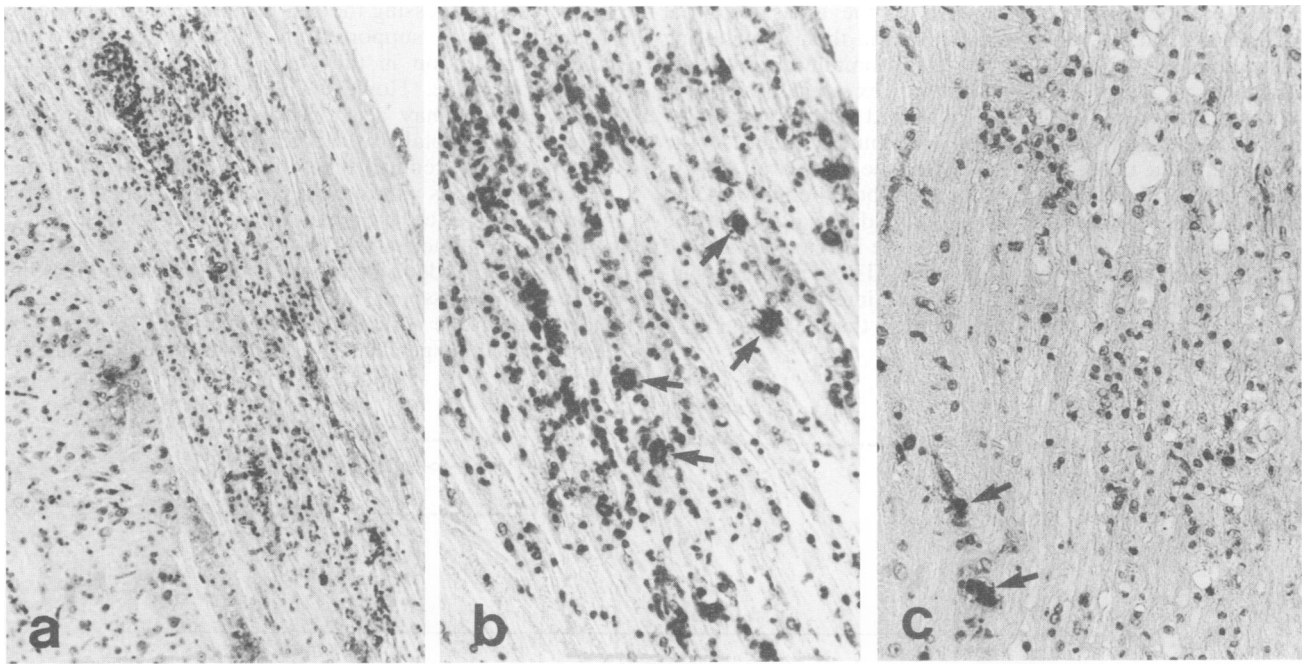

FIG. 6. Immunoenzyme staining of viral antigen in the spinal cord of the Ab+ group on days 10 and 20 p.i. Spinal cord sections from days 10 and 20 p.i. were stained with MAb J.3.3 to show the viral antigen distribution (indicated by arrows). (a) On day 10 p.i., white matter, showing the glial cells containing viral antigen and mononuclear cell infiltration, particularly in perivascular area. Magnification, ×350. (b) Area similar to that shown in panel a. Magnification, ×700. (c) On day 20 p.i., white matter lesion, showing cellular infiltrates and demyelination. Magnification, ×700.

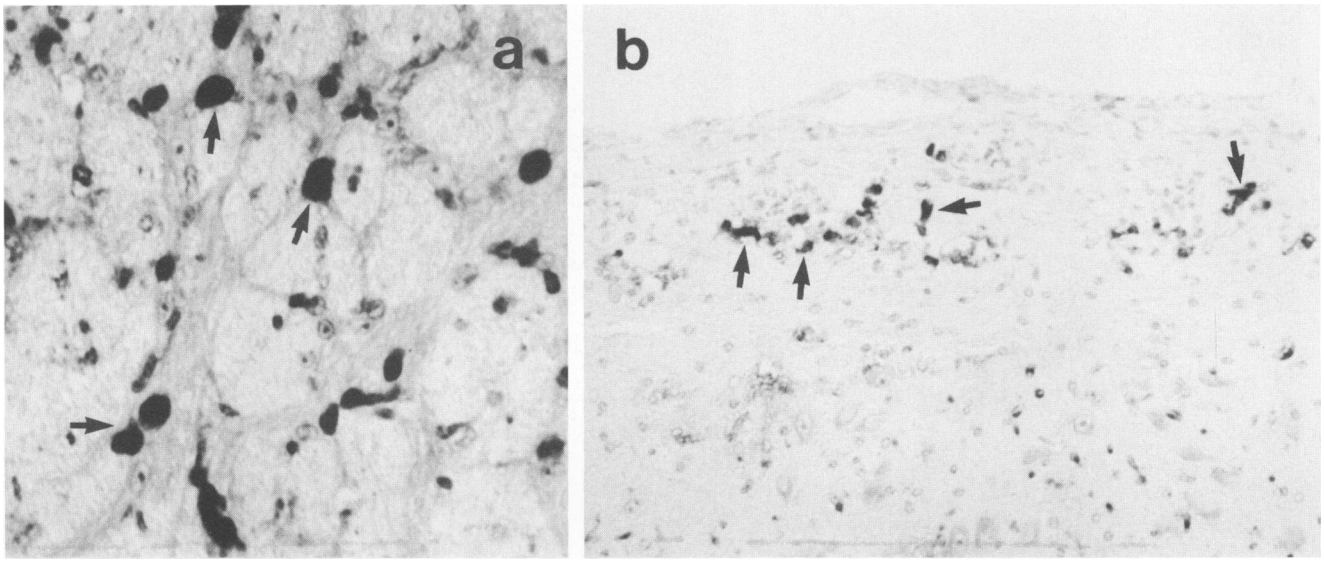

FIG. 7. Immunoenzyme staining of viral antigen in the brain and spinal cord of the Ab+ group on day 100 p.i. Brain and spinal cord were immunoenzyme stained with the MAb J.3.3. The area of severe typical demyelination and mild cellular infiltrates is shown. Viral antigen is indicated by arrows. (a) Basal ganglia. Viral antigen is in the glial cells (arrows) surrounding the axon. Magnification, ×1,400. (b) White matter of the spinal cord. Viral antigen is present in the oligodendrocytes, and demyelination and cellular infiltrates are prominent. Magnification, ×700.

viruses, so that viruses infect preferentially a particular cell type (2, 3, 11). In our current study, even in the presence of MAb, both JHM(2) and JHM(3) infected neurons as well as glial cells, indicating that the cellular tropism of the virus was not altered. However, early in infection, the distributions of viral antigen in immunized and nonimmunized mice were different. For example, on day 3 p.i., in immunized mice, viral antigen was restricted to the areas of initial inoculation, such as ependymal cells and their neighboring cells around the ventricles, while in nonimmunized mice, antigen was also detected in the cortex area. By day 5, however, the viral antigen was detected in the cortexes of immunized mice, similar to the distribution found in the nonimmunized mice on day 3. Likewise, the viral antigen was first observed in the spinal cords of both immunized and nonimmunized mice on the same day (day 5 p.i.), but the virus spread from ependymal cells to the interior of the cord was delayed in the immunized mice. These findings suggested that the HE-specific antibody interfered with the spread of virus in the brain, rather than altering viral tropism.

The finding that the virus spread was more restricted inside the brain tissue than within the cerebrospinal fluid was consistent with the fact that these MAbs did not neutralize viral infectivity. Thus, the major effect of HE-specific MAbs may be an accelerated activation of an innate immune mechanism, such as complement-mediated cytolysis or antibody-dependent cytotoxicity, which could clear virus-infected cells expressing the HE protein from the brain. This mechanism was supported by the finding of prominent cellular infiltration in the early stage of infection in the immunized mice. However, it is possible that the HE-specific MAbs may also opsonize the virus particles and facilitate their uptake by macrophages; as a result, the viral titer would be reduced and the spread of virus from the ependymal cells would be less rapid. This mechanism has been demonstrated in Semliki Forest virus-induced encephalitis (1). Both mechanisms may contribute to the reduction of virus titer. Indeed, we showed that the virus titers in the brains of the passively immunized mice were 1 $\log_{10}$ lower than those in the nonimmunized mice (Fig. 3), even though the HE protein-specific MAbs lack virus-neutralizing activ-

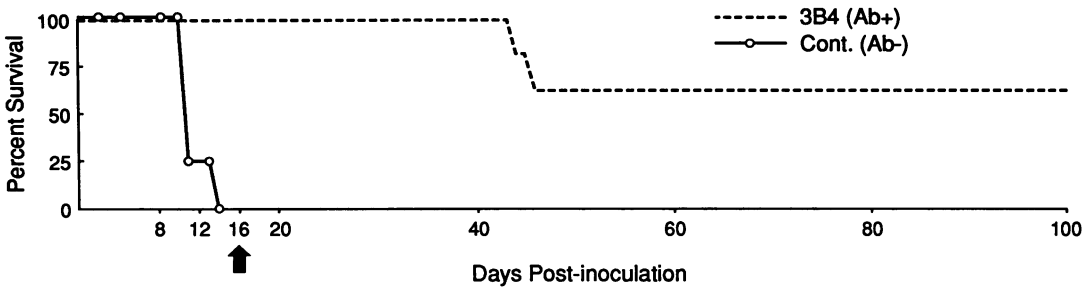

FIG. 8. Passive immunization of JHM(3)-infected mice with the HE-specific MAb. The mice were infected i.c. with JHM(3) (100 PFU) and inoculated i.p. with 100 μg of MAb 3B4 or the same volume of PBS. The mortality of these two groups is shown. Each group consisted of five mice. The arrow indicates the appearance of hind-leg paralysis in surviving mice.

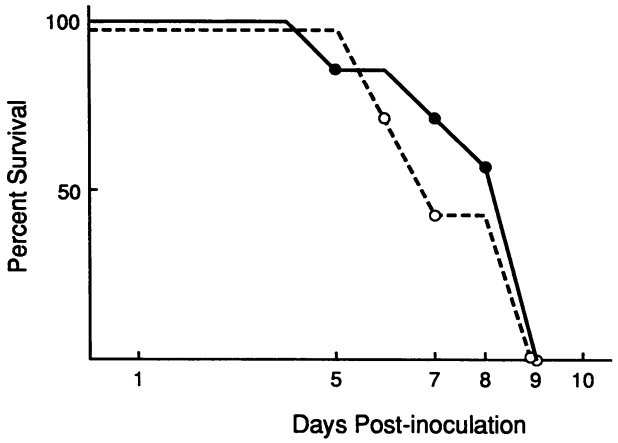

FIG. 9. Passive protection of At11f-infected mice with the HE protein-specific MAb. Inoculation of At11f virus (100 PFU) i.c. was immediately followed by i.p. injection of 100 μg of MAb 3B4 (Ab+ group, ●) or the same volume of PBS (Ab− group, ○). The mortality of the two groups is shown. For each group, $n = 7$.

ity in vitro. Thus, the mechanism of protection by HE-specific MAbs is more similar to that mediated by the S protein-specific neutralizing MAbs, which decreased viral titers (3), than to that mediated by the M or S protein-specific nonneutralizing antibodies, which appeared to alter the cellular tropism of the virus (3, 7). This finding is similar to the findings of passive immunization studies of other viruses (10, 11, 28, 32), which have shown that the protective capacity of MAbs did not correlate with the neutralization activity or Ig subclass of the antibodies.

With JHM infection of mice, antibodies against viral structural proteins were detected as early as day 7 (35a), signaling the beginning of the host's adaptive immune response, and correspondingly, virus was not found in neurons thereafter. In immunized mice, viral antigen was restricted to glial cells or monocytes in the spinal cord and not detectable in the brain on day 7 p.i. However, on day 10 p.i., virus titer in the brain increased again and, at least up to day 20 p.i., was maintained at the level of approximately 10^4 PFU/g of brain tissue (Fig. 3). This resurgence of virus titers in immunized mice could be the result of the decreased antibody titer, as suggested by a similar study with lactate dehydrogenase-elevating virus, in which reinjection of a protective MAb on day 7 p.i. increased the protective effects of the MAb (11). The timing of increase of MHV titers in the immunized mice corresponded to the clustering of the animals' deaths during days 20 to 30 p.i., probably as a result of recurring encephalomyelitis. We performed a study in which a second injection of the HE-specific MAb was administered on day 12 and found that mice were protected from death which otherwise occurred during the first window (data not shown). It is conceivable that the early inhibition of virus spread may have prevented complete induction of virus-specific immune response. These data also suggested that the passive immunization of HE-specific antibodies had a protective effect against JHM-induced encephalomyelitis, but not against demyelination.

On day 100 p.i., when infectious virus was no longer recoverable from the central nervous system, a significant amount of viral antigen remained in glial cells, with some mild demyelinating lesions in the brain, brain stem, and spinal cord (Fig. 7) (data not shown). This finding suggested that passive immunization with the anti-HE MAb resulted in a persistent infection by defective viruses, similar to the other models of JHM virus infection (16).

This study shows that passive immunization with HE protein-specific MAbs confers protection against MHV infection. The effect is similar to that seen with MAbs specific for the other three MHV structural proteins. Thus, although HE protein is a nonessential viral protein, its expression in the virus particle and/or the infected cells provides a target for the immune response. Furthermore, the HE-specific MAb, similar to some of the MAbs specific for other structural proteins of MHV, appears to alter spread or distribution of virus. Thus, the HE protein will likely contribute to viral pathogenesis in infections caused by MHVs which synthesize this protein. It is interesting to note that the ability to express HE protein is retained by most of the neurotropic strains of MHV, such as JHM, while its expression is lost in most hepatotropic MHV strains (36), although it is not essential for viral replication in vitro.

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