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A Neutralization-Resistant Theiler's Virus Variant Produces an Altered Disease Pattern in the Mouse Central Nervous System

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Theiler's murine encephalomyelitis virus infection of mice is an animal model for human demyelinating diseases. To further define the role of this virus in the disease process, we selected a virus variant resistant to neutralization by a monoclonal antibody to VP-1. This virus variant was then injected into SJL/J mice. Central nervous system tissue was compared between variant virus- and wild-type virus-infected mice. Within the brain, no large differences were observed between the two groups as to the distribution of inflammatory infiltrates around the injection site and the number of viral antigen-positive cells during the first weeks of the observation period. In contrast, in the spinal cord major differences were found between variant virus- and wild-type virus-infected mice regarding the number of inflammatory lesions, infected cells, and the size of the areas involved with time. By immunohistochemistry, equivalent numbers of infected cells could be found in the spinal cord 1 week postinfection (p.i.): however, after that time, the number of infected cells in the wild-type virus-infected mice continued to increase, whereas the virus-positive cells from the variant virus-infected mice gradually decreased. Thus, the number of viral antigen-containing cells peaked by 1 week p.i. in the variant virus-infected animals. Conversely, the number of infected cells in the spinal cords from mice inoculated with wild-type virus steadily increased until 8 weeks p.i. At this time (8 weeks p.i.), no more variant virus antigen-positive cells could be observed within the spinal cord. Plaque assay of central nervous system tissue confirmed these differences between the two groups observed by immunohistochemistry. No infectious variant virus could be isolated after 2 weeks p.i. from the brain and 4 weeks p.i. from the spinal cord, whereas infectious wild-type virus could be detected up to the end of the observation period (12 weeks p.i.). Virus which was isolated from variant virus-infected mice still retained the neutralization-resistant phenotype. These studies emphasize the important biological in vivo activity of Theiler's virus VP-1 in determining neurovirulence.

Theiler's murine encephalomyelitis virus (TMEV), a positive-strand RNA virus, is a naturally occurring murine picornavirus that leads to an asymptomatic enteric infection in young adult mice. A spontaneous paralytic central nervous system (CNS) disease has been observed in mice on rare instances (11). However, after intracerebral (i.c.) infection with the Daniels (DA) strain of TMEV in susceptible Swiss outbred mice, a consistent biphasic disease of the CNS was observed (18). In the acute stage of the infection, the animals showed the clinical symptoms of a poliomyelitis. Inflammation, necrosis of neurons, and glial cell proliferation were found to parallel virus replication. Surviving animals progressed to the chronic form of the disease 1 to 3 months postinfection (p.i.). In other studies analyzing the susceptibility of inbred mice strains, the acute disease was observed to be less severe in SJL/J mice than in the Swiss outbred mice (19). Most SJL/J mice survived the acute poliomyelitis without demonstrating severe clinical symptoms and developed a chronic disease. The pathological features included extensive meningeal and perivascular infiltrates with concomitant demyelination primarily in the spinal cord. These infiltrates consisted of lymphocytes, monocytes, some plasma cells, and macrophages (8). The white matter disease was reported to be similar to that observed in experimental allergic encephalomyelitis, an autoimmune CNS disease (7).

There is still a controversy about the mechanism of demyelination after TMEV infection—whether it is strictly virus induced or immune mediated. Early immunosuppression by cyclophosphamide diminishes the mononuclear cell infiltrates in the spinal cord and prevents demyelination (20). Immunosuppressive regimens altering demyelination are time dependent (28). Diminished demyelination has been reported after the administration of antibodies against major histocompatibility complex Ia antigen, suggesting that the observed demyelination after TMEV was, at least in part, mediated by the immune system. Other observations favor a mechanism of demyelination in which TMEV plays a central role. Demyelination has been associated with ongoing CNS TMEV infection (5). Viral RNA was always present at locations with ongoing demyelination. In addition, TMEV was found to persist in glial cells in the spinal cord (3). Viral antigen could still be detected in mouse spinal cord 2.5 years after infection (21). T-cell-independent demyelination was observed in nude mice after TMEV infection (29). Some of the infected cells in nude mouse CNS tissue have even been identified as oligodendrocytes by ultrastructural methods by demonstrating connection of cell bodies to myelin sheaths (30). By the use of combined immunohistochemistry and in situ hybridization techniques, virus antigen was observed within macrophages and astrocytes and 25 to 40% of the infected cells were identified as oligodendrocytes (2). Infection of oligodendrocytes, the myelin-producing cells, may support a direct virus-induced demyelinating mechanism by disturbing myelin metabolism. In contrast, it could also favor an immune-mediated mechanism through either a humoral or cellular immune response against TMEV antigen-presented oligodendrocytes (2).

TMEV has three major capsid proteins, VP-1, VP-2, and VP-3. VP-1 is the most external viral capsid protein and plays a central role in TMEV neutralization and pathogenesis. The largest number of nucleotide changes were observed

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within VP-1 when the sequence of the highly virulent GD-VII TMEV strain was compared with the less virulent BeAn strain (26). Furthermore, a major neutralizing epitope was localized on VP-1 (24) and different neutralizing monoclonal antibodies (MAbs) bound to VP-1 as measured by Western immunoblots (23). Thus, all the above-described functions suggest a biologically important role for VP-1 in TMEV infection.

Similarly, in the Sabin type 1 strain of poliovirus, another member of the picornaviruses, the dominant neutralizing domain was also localized within the largest, most exposed capsid protein, VP-1 (15). Regions of recombinant type 1 poliovirus VP-1 induced neutralizing antibodies when injected in rabbits (14), and isolated type 1 poliovirus VP-1 induced a neutralizing antibody response in rats (6). Synthetic peptides corresponding to residues of type 1 poliovirus VP-1 conjugated to carrier polypeptide could elicit neutralizing antibody (15). In addition, one of the important epitopes determining neurovirulence was localized within poliovirus VP-1, as shown by different recombinant studies with several types of polioviruses (1, 16, 25).

Recent observations have shown that antigenic mutants from various viruses can be selected with neutralizing MAbs (12, 22, 32). Some of the selected virus variants induce an altered pathogenicity when injected into animals. Neutralizing MAbs to reovirus type 3 hemagglutinin were used to select variants which were markedly less neurovirulent (31). Similarly, Fleming et al. (13) and Dalziel et al. (10) selected coronavirus variants resistant to neutralization by using MAbs to the E2 viral glycoprotein. These variants had a reduced neurovirulence when compared with infection with the wild-type virus.

Therefore, to define the role of TMEV VP-1, particularly that of the neutralizing determinants, in initiating CNS disease, we selected TMEV antigenic viral variants resistant to neutralization by using MAbs to VP-1. These virus variants were then inoculated i.c. into susceptible SJL/J mice. In the present study, we compared (i) the inflammatory infiltrates, (ii) the number of infected cells by immunohistochemistry, and (iii) virus titers over a period of 12 weeks in the CNS of mice infected with TMEV variants versus wild-type viruses.

MATERIALS AND METHODS

Cells. BHK-21 cells were cultured in Dulbecco modified Eagle medium (Irvine Scientific, Santa Ana, Calif.) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah), nonessential amino acids, glutamine, sodium pyruvate, penicillin, streptomycin, and amphotericin B (Fungizone). Cells were split twice a week.

Virus. The DA strain of TMEV was propagated in BHK-21 cells in Dulbecco modified Eagle medium. The DA virus was originally obtained from J. Lehrich and B. Arnason (17). Our DA stock was derived from this stock by J. L. Leibowitz. The DA virus used in this study was plaque purified three times. A virus pool was prepared in BHK-21 cells, and the virus variants were generated from this pool. This stock virus pool was 3.7×10^7 PFU/ml.

MAbs used for variant selection. Two different Mabs, H7 and H8, were used for variant selection. The Mabs have been characterized by Western immunoblots with purified TMEV proteins separated by 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13a). Both react with VP-1, and in a 50% endpoint neutralization assay, MAb H7 had a titer of 1/250,000 and MAb H8 had a titer of 1/10,000. By competitive inhibition and Western blotting assays, these Mabs bound to close yet distinct epitopes (13a). Ascites fluid from these MAbs was prepared by injecting the hybridomas into pristane-treated BALB/c mice.

Selection of virus variants. DA virus (10^7 PFU) in a volume of 900 μl plus 100 μl of neutralizing ascites fluid was incubated on ice for 1 h. Various dilutions of the virus-antibody mixture were then absorbed onto BHK-21 cells for 1 h at 37°C. Cells were then overlaid with 0.5% agarose in medium 199 containing 1% fetal bovine serum and incubated for 4 days at 37°C. Virus which escaped neutralization formed single plaques, which were picked and diluted in medium containing 10% neutralizing MAb and incubated for 1 h on ice. This neutralization assay was repeated twice more. After the last plaque purification, a stock of the virus variant was prepared in BHK-21 cells and the virus titer was determined by a conventional plaque assay. The variant virus pools were 3.8×10^7 PFU/ml for H7 and 1.0×10^7 PFU/ml for H8.

Mice and infection. For this study, 4- to 6-week-old SJL/J mice (breeding colony, Research Institute of Scripps Clinic, La Jolla, Calif.) were used. In a pilot experiment, SJL/J mice (five mice per group) were infected i.c. with a concentration of 5×10^5, 5×10^3, or 5×10^1 PFU of selected DA variant viruses in 50 μl of phosphate-buffered saline. As a positive control, SJL/J mice were infected with the same concentrations of the wild-type DA virus stock used for the variant selection. Four groups were infected with variants selected by MAb H8. All four variant viruses selected with MAb H8 appeared similar to the parental DA wild-type virus when compared by immunohistochemistry and histopathology after i.c. inoculation of mice. Six groups were infected with MAb H7-selected plaque-isolated variants. Three of these H7 variant virus-infected groups had a pattern of a disease similar to that of mice infected with the parental DA wild-type virus, and the three other groups had clearly diminished disease compared with mice infected with the parental DA wild-type virus after i.c. inoculation. A variant virus, H7A6-2, which showed a markedly altered pathogenicity 6 weeks after infection compared with the wild type virus was chosen for further study. This variant virus was then inoculated i.c. into 70 SJL/J mice at a concentration of 3×10^5 PFU. Another group of mice was infected with the same concentration of wild-type virus. In addition, a group of mice was inoculated i.c. with supernatant fluid from uninfected, freeze-thawed, lysed BHK-21 cells and served as negative controls. Ten mice from each group were sacrificed at 3 days and 1, 2, 4, 6, 8, and 12 weeks p.i.

Plaque assay. TMEV was quantified by plaque assay on BHK-21 cells. Five mice from each time point were euthanized, and CNS tissues were obtained aseptically, transferred to preweighed tubes, and homogenized in phosphate-buffered saline. The resulting tissue homogenates were then freeze-thawed three times. Dilutions of homogenized tissues as well as control TMEV were then added to cell monolayers in 35-mm plastic wells (Costar, Cambridge, Mass.). The tissue homogenates or control virus were allowed to absorb for 1.5 h at room temperature, after which cells were overlaid with 0.5% agarose in medium 199 containing 1% fetal bovine serum. After 4 days at 37°C, cell monolayers were fixed and stained with crystal violet and plaques were enumerated. The limit of detection of the plaque assay was 5 PFU of wild-type virus per ml.

Virus isolation. Isolated virus from brains and spinal cords from mice infected with the wild-type DA virus or variant H7A6-2 was plaque purified, and a virus stock was prepared

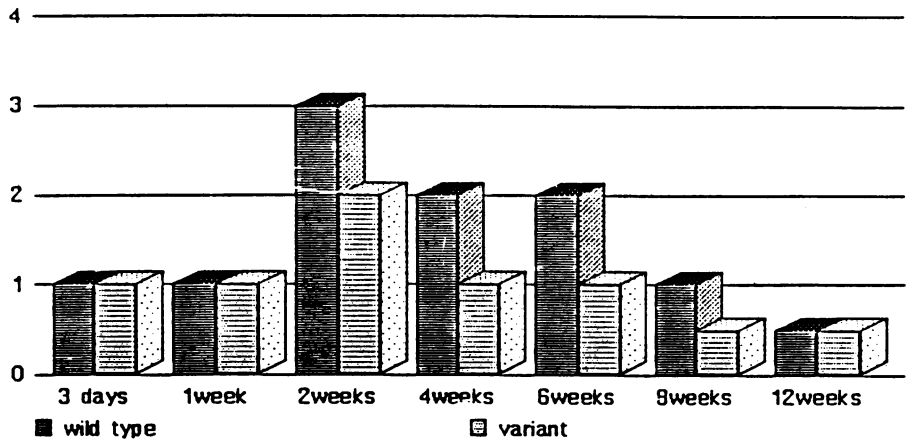

FIG. 1. Kinetics of inflammation within the brain from animals infected with wild-type virus versus variant virus. Number and size of inflammatory lesions are compared on a scale from 1 to 4. 1 equals 2 to 3 small lesions per whole brain section, and 4 equals more than 20 large inflammatory lesions or confluent areas of inflammation within the brain. No marked difference was observed within the brain.

as described above. After the virus titers were determined, a neutralization assay was performed with MAbs H7 and H8 and rabbit anti-DA antiserum (described below) with two plaque isolates each.

Animal tissue. Five animals from each time point were perfused with 4% phosphate-buffered saline-buffered paraformaldehyde (J. T. Baker Chemical Co., Phillipsburg, N.J.). Brains and spinal cords were removed and processed for paraffin embedding. Sagittal sections of 4 μm were cut and stained with hematoxylin and eosin for histologic study. Corresponding sections were used for immunocytochemistry. The remaining five mice per time point were anesthetized with Metofane (methoxyflurane; Pitman-Moore, Inc., Washington Crossing, N.J.), blood was collected, the mice were euthanized, and CNS tissue was prepared for plaque assay.

Antibodies for immunocytochemistry. Anti-DA antibodies were raised in rabbits by intravenous immunization with purified virus every fourth week for 6 months. The serum was tested by enzyme-linked immunosorbent assay and Western immunoblots. Sera had titers greater than 1/150,000 by enzyme-linked immunosorbent assay (27) and reacted strongly with VP-1 and VP-2 and weakly with VP-3 by Western blotting.

Immunocytochemistry. Immunocytochemistry was performed as previously described (33). Briefly, deparaffinized sagittal brain and spinal cord sections from mice infected with variant virus or wild-type virus were treated with 5% normal goat serum (Sigma Chemical Co., St. Louis, Mo.), followed by the rabbit anti-DA antiserum in a dilution of 1/8,000 overnight at 4°C. Then the slides were incubated consecutively with goat anti-rabbit immunoglobulin G (Tago, Burlingame, Calif.) in a dilution of 1/50, avitin biotinylated horseradish peroxidase as prescribed by the vendor (Vector Laboratories, Burlingame, Calif.), diaminobenzidine, and hydrogen peroxide. After the reaction was stopped by washing the slides with phosphate-buffered saline, the slides were dehydrated, cover slipped, and examined by light microscopy.

RESULTS

Histopathology. To study the development of inflammatory lesions within the CNS, we obtained sagittal brain and spinal cord tissue at 3 days to 12 weeks p.i. Sagittal brain and spinal cord sections were then stained with hematoxylin and eosin and examined under a light microscope for the presence of inflammatory cells. Early after infection, the distribution of the lesions within the brain was similar irrespective of what virus (wild-type virus versus variant virus) was used to infect the mice (Fig. 1). Three days p.i., a large hemorrhagic lesion and a few small perivascular cuffs immediately adjacent to the injection site were evident. No histopathologic abnormalities could be detected in the spinal cord at this time. After 1 week, marked lesions were present at the injection site in the brain, with other inflammatory lesions distributed throughout the medulla, midbrain, and hippocampus. These lesions consisted of degenerating neurons, neuronophagia, microglial nodules, and perivascular infiltrates composed of mononuclear cells, including lymphocytes, monocytes, and plasma cells. In addition, inflammatory infiltrates could be observed within the meninges and in perivascular areas in the white matter, with a few small perivascular cuffs within the grey matter of the spinal cord. The inflammation appeared to spread from these perivascular cuffs and leptomeninges to the adjacent white matter. With time, the number and size of the inflammatory lesions declined in the brain (Fig. 1).

Although no marked difference in the distribution of lesions (Fig. 2a and b) was observed in the brain, a contrasting pattern in the spinal cord regarding size, number, and time course of the inflammatory lesions was noted (Fig. 2c and d). The first histological difference could be readily observed by 1 week after infection (Fig. 3). Mice infected with variant virus had a few more and larger inflammatory lesions in the spinal cord than the wild-type virus-infected animals. The inflammatory response in the variant virus-infected mice peaked by 2 weeks p.i. and then gradually declined. In contrast, the lesions in the wild-type virus-infected animals grew in size and number until approximately 2 months after infection. In addition, the inflammatory lesions in the spinal cord of the variant virus-infected mice never reached the extent of those observed in wild-type virus-infected animals (Fig. 3). This difference between wild-type virus- and variant virus-infected mice was most pronounced by 6 to 8 weeks p.i. At that time, animals infected with wild-type virus had extensive inflammatory lesions in the spinal cord, whereas only a few small perivas-

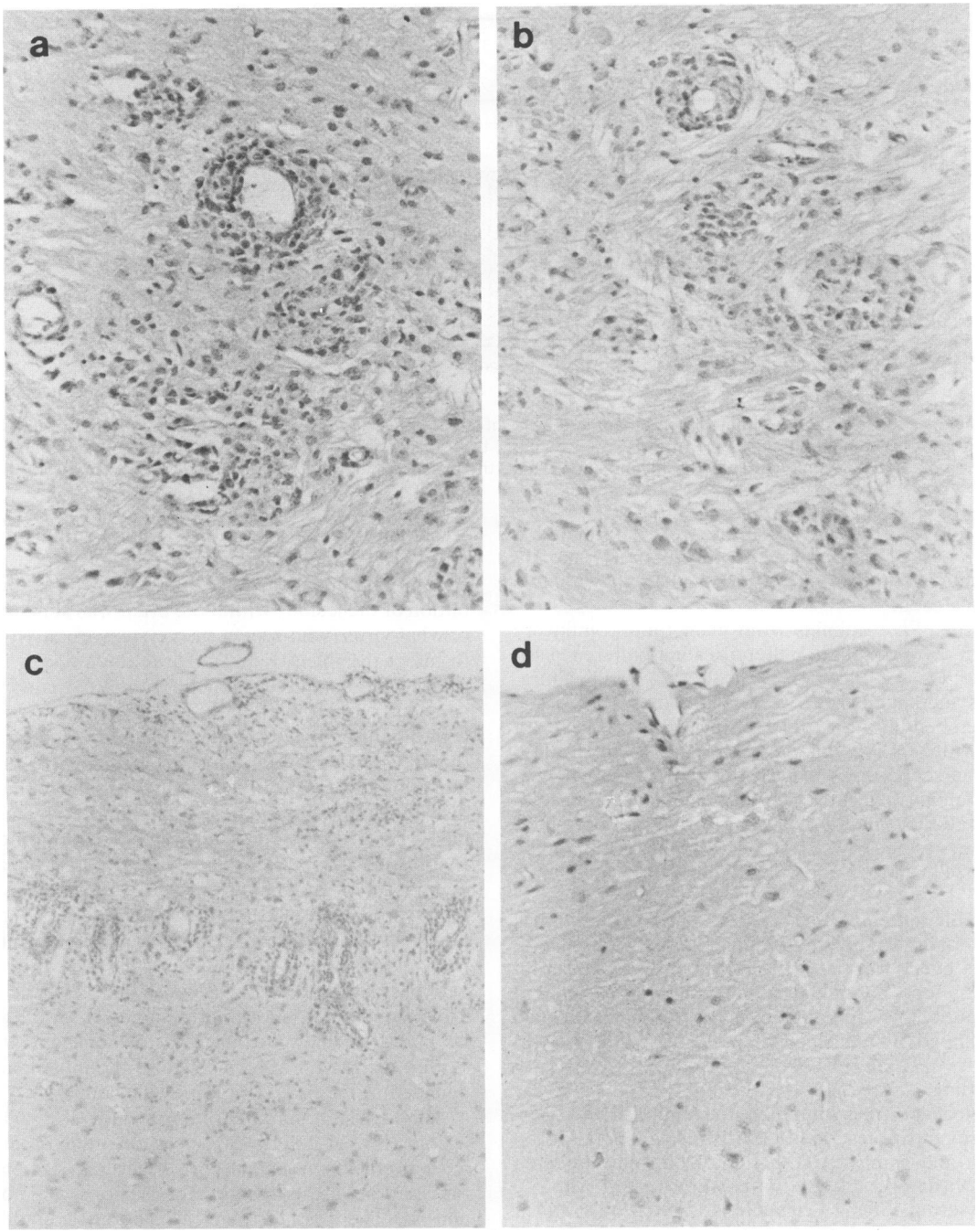

FIG. 2. (a) Inflammatory lesions with perivascular cuffs in the brain from wild-type virus-infected mice 2 weeks p.i. (b) Inflammatory lesions with perivascular cuffs in the brain from variant virus-infected mice 2 weeks p.i. (c) Large perivascular cuffs in the spinal cord from mice infected with wild-type virus at 6 weeks p.i. (d) No inflammatory lesions can be observed in the spinal cord of this mouse 6 weeks after infection with variant virus. All panels show hematoxylin and eosin staining, ×250.

cular cuffs could be found in the spinal cord from variant virus-infected mice.

Viral antigen distribution. To quantify the amount of TMEV protein in the CNS, we demonstrated the presence of viral antigen by immunocytochemistry with rabbit anti-DA virus antiserum. The viral distribution in brains and spinal cords from wild-type virus- and variant virus-infected mice was similar (Fig. 4a and b). Many of the viral antigen-positive cells were localized in and around the injection site. Concomitantly, the pyramidal cells and their processes in the hippocampus contained viral antigen. The number of infected cells in the CNS 3 days after infection in wild-type virus- or variant virus-infected mice was similar (Table 1). At this time point, more than 100 infected cells could be detected in the brain sections from wild-type virus- and variant virus-infected animals. By 1 week p.i., considerably less viral antigen was localized in and around the large inflammatory lesion at the injection site. The number of infected brain cells steadily declined. A drop in number of infected cells within the brain in the variant virus-infected

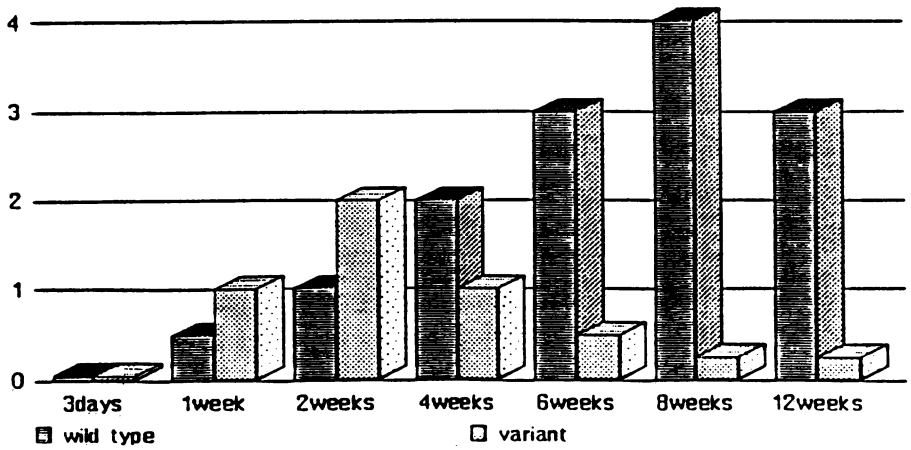

FIG. 3. Kinetics of inflammation within the spinal cord from animals infected with wild-type virus versus variant virus. Number and size of inflammatory lesions are compared on a scale from one to four. One equals 2 to 3 small lesions per longitudinal spinal cord section, and four equals more than 20 large inflammatory lesions or confluent areas of inflammation within the spinal cord. The inflammatory lesions in the spinal cords of the variant virus-infected mice peaked by 2 weeks p.i. and never reached the extent of that observed in wild-type virus-infected animals.

mice was observed at 2 weeks p.i. In the wild-type virus-infected animals, a decrease of infected cells was observed at 4 weeks p.i. This level was maintained throughout the observation period. At 2 weeks p.i., a small difference in number of infected cells in wild-type virus-infected mice versus variant virus-infected mice was observed. No substantial difference was demonstrated between these groups of animals at 4 weeks p.i. Brains from mice infected with the wild-type virus for 8 weeks still contained a small number of infected antigen-positive cells (10 to 20), whereas no variant virus-infected cells could be found. The decrease in the number of infected cells in the brains of variant virus-infected mice from 1 to 2 weeks p.i. occurred in all observed mice and may reflect immune elimination of infected cells.

In the spinal cords from wild-type virus-infected versus variant virus-infected mice, a marked difference in the number of cells containing viral antigen was seen from 2 weeks p.i. (Table 1). Viral antigen was present in glial cells, macrophages, and single neurons mainly within and around the inflammatory infiltrates. The pattern of viral antigen distribution was similar, but the number of infected cells differed between the groups. At 3 days p.i., no viral antigen-positive cells in spinal cord could be detected in either group. However, beginning at 1 week p.i., single cells that were positive for viral antigen were present in wild-type virus- and variant virus-infected mice. The number of infected cells in the variant virus-infected spinal cord peaked at 1 week p.i. and then gradually declined. By 2 weeks p.i., only a few infected cells were present in the spinal cords of variant virus-infected animals, whereas the number of wild-type virus-infected cells slowly increased until 8 weeks p.i. By 12 weeks p.i., viral antigen could no longer be detected in the spinal cords of variant virus-infected mice. At this time point, the amount of virus antigen had also decreased in the wild-type virus-infected animals; however, there was still a clear difference between both groups by the end of the observation period (Table 1). Thus, there was a clear difference in the amount of viral antigen-containing cells within the spinal cord between wild-type virus- and variant virus-infected mice (Fig. 4c and d).

Infectious virus. To determine the amount of infectious virus present and to correlate this with number of infected and antigen-positive cells, we plaqued brain and spinal cord tissue homogenates from wild-type virus- and variant virus-infected mice on BHK-21 cells (Table 2). Similar amounts of virus in brain tissue from wild-type virus- and variant virus-infected mice were detected at 3 days p.i. (Table 2). At 1 week p.i., about 50 times fewer PFU were detected in brains and spinal cords from variant virus-infected mice than from wild-type virus-infected animals. At 2 weeks p.i., no detectable virus could be isolated from the brains of variant virus-infected animals, whereas the mice infected with wild-type virus still contained detectable infectious TMEV particles. About 10^5 PFU/g of tissue could be detected in spinal cord tissue from both groups at 3 days p.i. (Table 2). In the variant virus-infected mice, the amount of infectious virus in spinal cord steadily declined. At 2 weeks p.i., four of five mice contained no detectable virus, and at 4 weeks p.i., no more virus could be demonstrated by a plaque assay. In contrast, spinal cord tissue from mice infected with the wild-type virus contained a relatively constant number of infectious viral particles, and at 8 weeks about 2×10^4 PFU/g of spinal cord was still detected (Table 2). Virus isolated from mice infected with wild-type DA was neutralized by the rabbit anti-DA antibody and by both MAbs H7 and H8. However, virus (two plaques were picked) that was isolated from H7 variant-infected brains and spinal cords was neutralized only by MAb H8 and the rabbit antiserum but not by MAb H7. Therefore, isolated H7 variant viruses retained the H7 resistant phenotype.

DISCUSSION

This report analyzed the pattern of disease induced in mice infected with a variant of TMEV. This variant was selected by using a neutralizing MAb against the capsid protein VP-1. This is the first demonstration that altering TMEV VP-1 could change the extent and degree of pathology in the CNS. Nucleic acid sequence comparisons between virulent and less virulent TMEV strains have shown that the largest number of changes occur in the VP-1-coding region, supporting the biological importance of VP-1 for virulence (26). Other investigations (1, 6, 14–16, 25) have underlined the importance of VP-1 for neutralization and for contributing to the virulence of poliovirus, another picornavirus.

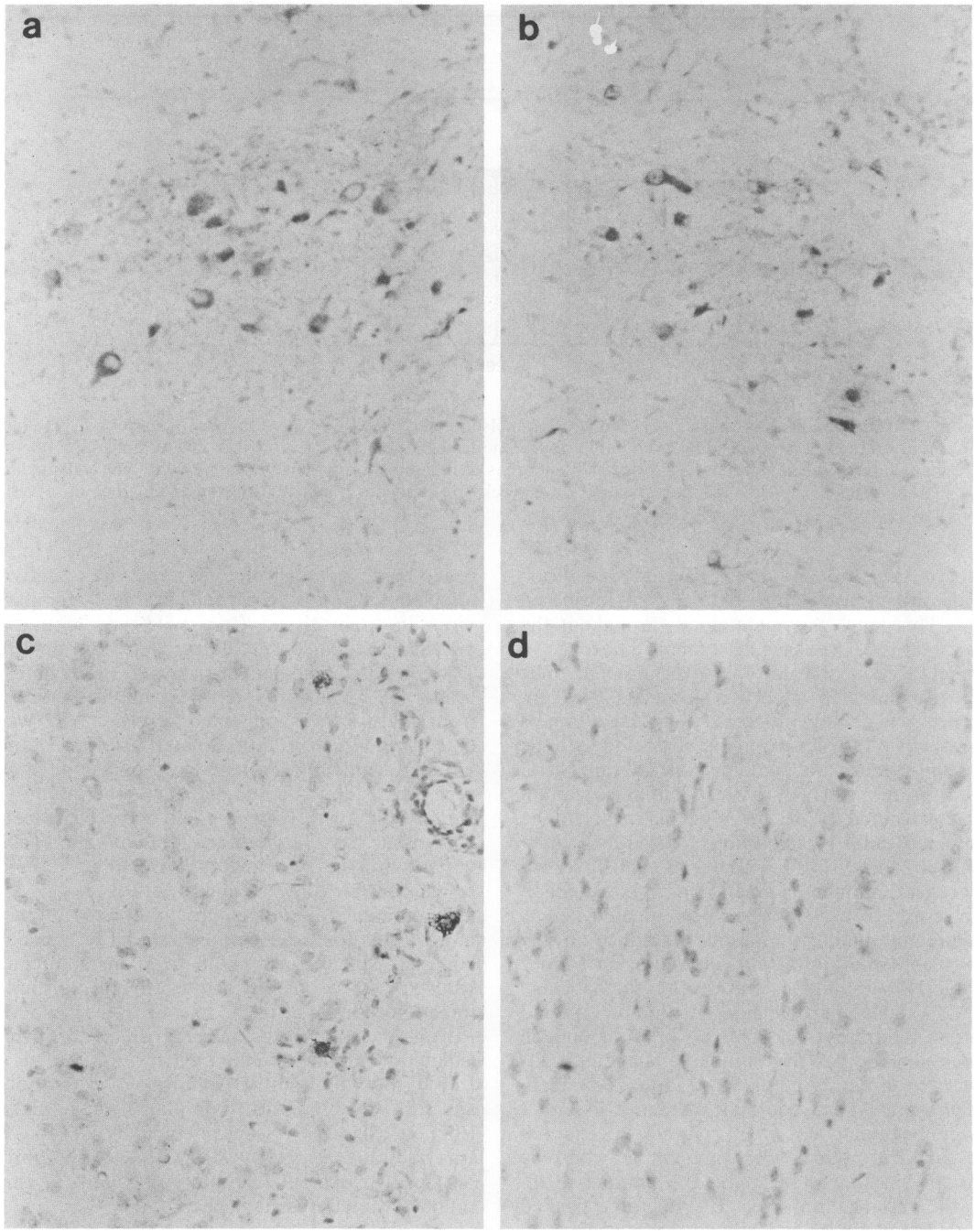

FIG. 4. (a) TMEV antigen in the cytoplasm of wild-type virus-infected brain cells 2 weeks p.i. (b) TMEV antigen in the cytoplasm of variant virus-infected brain cells 2 weeks p.i. (c) TMEV antigen in the cytoplasm of wild-type virus-infected cells in the grey matter of the spinal cord 6 weeks p.i. (d) No TMEV antigen can be detected in the spinal cord of variant virus-infected mice by 6 weeks p.i. All panels were stained with anti-DA–avidin peroxidase, ×250.

To study the spread and kinetics of replication, we injected similar amounts of wild-type and variant virus i.c. into SJL/J mice. CNS tissue from these infected animals was compared for number and size of lesions, number of infected cells, and the presence of infectious virus. Using these mice, we demonstrated that within the brain there were no marked differences between wild-type virus- and variant virus-infected mice regarding inflammatory infiltrates and only small differences regarding the number of viral antigen-containing cells during the first weeks of the observation period. In contrast, major differences in the number of infected cells as well as in the size and amount of inflammatory lesions were detected in the spinal cord. The selected variant virus caused much less inflammation in the spinal cord than the wild-type virus. The number of viral antigen-containing cells peaked in the variant virus-infected mice by 1 week p.i. and never reached the high numbers found in wild-type virus-infected mice, which steadily increased until 8 weeks p.i. in the spinal cord (Table 1). Also, the number of infectious virus particles isolated from CNS differed between wild-type virus- and

TABLE 1. Comparison of number of infected cells in CNS tissue from DA wild-type virus and variant (H7A6-2) virus-infected mice[a]

Time p.i.	Mouse no.	No. of infected cells			
		Wild-type virus		H7A6-2	
		Brain	Spinal cord	Brain	Spinal cord
3 days	1	>100	0	>100	0
	2	>100	1	>100	0
	3	>100	0	>100	0
	4	>100	0	>100	0
	5	>100	0	>100	0
	Avg	>100	0.2	>100	0
1 wk	1	16	6	26	0
	2	40	10	50	4
	3	90	3	80	0
	4	80	30	70	8
	5	40	3	20	17
	Avg	53.2	10.4	49.2	5.8
2 wks	1	40	50	1	0
	2	6	6	0	2
	3	4	26	2	0
	4	6	40	0	0
	5	6	21	0	1
	Avg	12.4	28.6	0.6	0.6
4 wks	1	19	64	4	0
	2	8	62	12	0
	3	0	58	4	1
	4	3	23	3	2
	5	9	20	4	0
	Avg	7.8	45.4	5.4	0.6
6 wks	1	12	98	5	1
	2	50	85	12	0
	3	8	13	0	5
	4	13	43	0	0
	5	35	63	0	1
	Avg	23.6	60.4	3.4	1.4
8 wks	1	9	49	0	1
	2	14	66	0	0
	3	14	96	0	0
	4	23	91	0	0
	5	8	38	0	2
	Avg	13.6	68	0	0.6
12 wks	1	15	40	0	0
	2	10	25	0	0
	3	11	23	0	0
	4	7	8	0	0
	5	4	7	0	0
	Avg	9.4	24	0	0

[a] Determined by immunohistochemistry.

TABLE 2. Comparison of infectious virus isolated from CNS tissue from wild-type virus- and variant (H7A6-2) virus-infected mice

Time p.i.	Mouse no.	PFU/g			
		Wild-type virus		H7A6-2	
		Brain	Spinal cord	Brain	Spinal cord
3 days	1	2.5×10^6	3.6×10^5	2.8×10^6	4.1×10^4
	2	4.0×10^6	8.7×10^4	4.5×10^6	9.9×10^4
	3	4.7×10^6	5.4×10^5	4.9×10^6	1.0×10^5
	4	6.7×10^6	4.3×10^5	5.3×10^6	2.5×10^5
	5	3.8×10^6	1.1×10^5	5.9×10^6	4.0×10^4
	Avg	4.3×10^6	3.1×10^5	4.7×10^6	1.1×10^5
1 wk	1	4.7×10^5	8.1×10^5	2.0×10^4	7.8×10^3
	2	3.2×10^5	6.1×10^4	3.5×10^4	2.7×10^3
	3	1.5×10^5	9.7×10^4	5.5×10^3	1.4×10^3
	4	3.6×10^5	3.4×10^5	3.7×10^4	1.0×10^4
	5	6.7×10^6	9.4×10^4	Not done	Not done
	Avg	1.6×10^6	2.8×10^5	2.4×10^{4a}	5.6×10^{3a}
2 wks	1	1.2×10^3	2.5×10^4	0	0
	2	3.4×10^3	1.4×10^4	0	0
	3	1.9×10^3	7.5×10^4	0	0
	4	1.4×10^3	8.0×10^4	0	0
	5	1.1×10^3	1.3×10^4	0	1.1×10^3
	Avg	1.8×10^3	4.1×10^4	0	2.1×10^2
4 wks	1	1.9×10^3	1.7×10^5	0	0
	2	1.7×10^3	1.9×10^4	0	0
	3	5.2×10^2	7.0×10^3	0	0
	4	2.7×10^3	2.1×10^3	0	0
	5	Not done	Not done		
	Avg	1.7×10^{3a}	5.5×10^{4a}		
6 wks	1	1.6×10^3	4.8×10^3	0	0
	2	0	5.1×10^3	0	0
	3	2.3×10^3	5.2×10^3	0	0
	4	3.3×10^2	6.9×10^3	0	0
	5	0	3.0×10^3	0	0
	Avg	8.6×10^2	5.0×10^3	0	0
8 wks	1	2.3×10^3	3.9×10^4	0	0
	2	1.2×10^3	2.1×10^4	0	0
	3	0	1.1×10^4	0	0
	4	8.1×10^2	1.6×10^4	0	0
	5	1.6×10^3	2.5×10^4	0	0
	Avg	1.2×10^3	2.3×10^4	0	0
12 wks	1	6.7×10^2	3.3×10^3	0	0
	2	9.7×10^2	3.4×10^3	0	0
	3	2.4×10^3	5.8×10^3	0	0
	4	9.6×10^2	4.5×10^3	0	0
	5	Not done	Not done	0	0
	Avg	1.2×10^{3a}	4.6×10^{3a}	0	0

[a] Average of four mice.

variant virus-infected mice. No infectious variant virus could be isolated after 2 weeks p.i. from the brain and after 4 weeks p.i. from the spinal cord, whereas infectious TMEV particles could be demonstrated up to the end of the observation period in wild-type virus-infected mice. The number of infected cells differed from the virus titers in wild-type virus- and variant virus-infected mice as compared in Tables 1 and 2. The highest titers in the spinal cord for both groups were found at 3 days p.i. At this time point, little or no viral antigen could be demonstrated by immunohistochemistry. However, the techniques measure different endpoints. The plaque assay detects infectious virus, which may not neces-

sarily be representative of antigen-positive cells. Further, circulating as well as cell-associated virus would score as positive with the viral plaque assay. Immunocytochemistry would not identify cell-free virus. Viral antigen within infected cells does not necessarily reflect the amount of infectious virus. These differences could explain why the two methods do not correlate.

The differences observed in the spinal cord could be attributed to viral replication or cell tropism. Replication of the variant virus within the spinal cord appeared to be restricted. However, variant virus replication was certainly not a general defect. Variant virus had a similar time course of replication and achieved comparable high titers in tissue culture cells (BHK-21) (data not shown) compared with wild type. Other virus variants selected in a similar fashion have produced an altered pathogenicity when injected into animals. Spriggs and Fields (31) selected reovirus type 3 variants using neutralizing MAb to the viral hemagglutinin. These mutants were markedly less neurovirulent but only grew in restricted sites within the brain. To determine whether a similar restriction occurs with the variant virus, we are currently examining growth kinetics of wild-type and variant viruses in various murine CNS-derived cell types. In general, tropism does not appear to be different since antigen was detected in comparable areas within the CNS.

The question why this variant is less neurovirulent remains open. The virus variant may be cleared more efficiently and also faster by the immune system because of important changes at the VP-1 region. TMEV-infected mice produce neutralizing antibodies (20). However, these antibodies may not limit virus spread efficiently, since infection with wild-type virus leads certain mice strains, such as the SJL/J mice, to develop a chronic disease with virus persistence in the spinal cord. It is conceivable that these antibodies recognize and/or neutralize our virus variant more efficiently, or it may be a question of timing. Conformational changes affecting one neutralizing site could expose another neutralizing site at a distal region of the structure. The virus may have already spread to and infected cells within the spinal cord prior to sufficient neutralizing antibody production. This could explain our finding that similar numbers of infected cells in the spinal cord can be found 1 week p.i. after infection with wild-type and variant virus. Virus has been reported to spread from the brain to the spinal cord by axonal and dendritic flow (9). In a recent report, we demonstrated viral RNA in cells associated with vascular endothelium in the CNS of TMEV-infected mice, suggesting an additional pathway of spread for TMEV (33). Variant virus could be limited or less efficient in cell-cell spreading, whereas wild-type virus is not.

Besides being cleared faster by the immune system, the variant virus, which is altered within the VP-1 region, may induce a persistent infection with only minimal viral antigen expression earlier than the wild-type virus does. Nitayaphan et al. (24) have suggested that disruption of a major neutralization epitope through VP-1 cleavage is important for virus persistence. Further, during the chronic stage of the disease, TMEV replication is restricted at the RNA level and no or only a few viral capsid proteins can be detected (4). Thus, this could help explain why detection of these infected cells by immunohistochemistry did not occur at later time points. Expressing less viral antigen could also explain the diminished inflammation that parallels the number of infected cells.

Although we found clear differences in the amount of viral antigen-containing cells and inflammatory lesions in the spinal cord, we did not observe any major differences in virus distribution within the CNS suggesting a similar tropism. Even the small amounts of variant viral antigen which were demonstrated by immunohistochemistry were found in the same areas as the wild-type virus antigen. However, variant virus never replicated in the spinal cord to the same extent as the wild-type virus. Therefore, a direct comparison of virus distribution within the CNS at later time points was not possible by immunocytochemistry. Coronavirus variants resistant to neutralization by MAb to the E2 viral glycoprotein were less neurovirulent than the wild-type virus (10, 13), suggesting that an altered cell tropism for the mutant virus was responsible for reduced neurovirulence. Our data are not consistent with changes in cell tropism of the variant virus when compared with the wild-type virus resulting in the altered pathology. However, to unequivocally determine changes in cell tropism, double-labeling studies by combined immunohistochemistry or in situ hybridization to identify wild-type virus- or variant virus-infected cells need to be performed and comparisons made at the initial stages of infection. These studies are necessary to specify any modifications in cell tropism and are under way.

We describe here a virus mutant selected by a neutralizing MAb which clearly induces an altered pathogenicity. This virus variant is most likely modified within the VP-1 region. Our observations emphasize the important biological activity of this epitope in vivo in determining neurovirulence.

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