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Survival of Athymic (*nu/nu*) Mice after Theiler's Murine Encephalomyelitis Virus Infection by Passive Administration of Neutralizing Monoclonal Antibody†

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Little or no antiviral immune response is mounted in athymic nude mice infected with the Daniels strain of Theiler's murine encephalomyelitis virus. In these athymic mice, increasing levels of infectious virus could be detected in the central nervous system. Seventy-five percent (9 of 12) of the nude mice were moribund or dead by 4 weeks postinfection. In contrast, treatment of Theiler's virus-infected nude mice with a neutralizing monoclonal antibody (H7-2) against the viral protein VP-1 resulted in a dramatic reduction of infectious virus within the central nervous system. All antibody-treated nude animals survived beyond 4 weeks postinfection. Monoclonal antibody titers could be maintained by passive transfer in treated nude mice at levels comparable to those of polyclonal antibody titers found in heterozygous infected *nu/+* littermates. Areas of demyelination were detected in the untreated animals as early as 7 days after infection with little or no remyelination present. In approximately one-half of the antibody-treated nude animals, no demyelinating lesions were found. However, the rest of these treated mice were found to have areas of both demyelination and remyelination. Thus, anti-Theiler's murine encephalomyelitis virus antibody against VP-1 can play a dramatic role in the survival of mice, clearance of virus, limiting viral spread, and altering the pattern of disease in the absence of a functional T-cell response.

In its natural murine host, intracerebral infection with Theiler's murine encephalomyelitis virus (TMEV) results in a chronic demyelinating disease associated with a persistent infection of the central nervous system (CNS) (9, 11). This experimental system has been used as a model for human demyelinating diseases (5). The contribution of a humoral antiviral immune response toward limiting TMEV spread and histopathology and promoting survival has yet to be defined, although immunocompetent mice do produce an antibody response to various viral components (10, 12). These studies suggest that the type of antibody response may influence the outcome of viral clearance, i.e., persistence. In addition, cellular responses have been well characterized and the T-cell phenotype involved in such responses has been analyzed (1, 2, 13).

Previously, we have demonstrated that athymic nude (*nu/nu*) mice which have an immature T-cell population fail to mount either a cellular or a humoral antiviral response when infected with TMEV (18). Furthermore, both viral proteins and nucleic acid can be found in neurons and in glial and endothelial cells in the CNS of nude mice (22). Recently, we have developed several monoclonal antibodies (MAbs) against the Daniels strain of TMEV (7). Some of these MAbs have the ability to neutralize TMEV in vitro. One of these MAbs (H7-2) was used to select neutralization-resistant variants which induce an altered pattern of disease when injected into mice (23).

The purpose of this study was threefold: (i) to delineate the relative contribution of the antiviral humoral response, i.e., antibody to the viral protein VP-1, in viral clearance during TMEV infection; (ii) to determine whether MAb against a single viral epitope can protect mice; and (iii) to assess whether treatment with MAb can change the pattern of disease.

MATERIALS AND METHODS

Cells and virus. BHK-21 cells were propagated in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, nonessential amino acids, glutamine, sodium pyruvate, penicillin, streptomycin, and amphotericin B (Fungizone). Cells were split twice weekly. The Daniels strain of TMEV was originally obtained from J. Lehrich and B. Arnason (8). Passage history, plaque purification, and amplification of the virus have been previously described (18).

Anti-TMEV MAb preparation. Hybridoma cells producing monoclonal anti-TMEV MAb (designated H7-2) were initially propagated in RPMI 1640 medium supplemented with glutamine, antibiotics, and 10% fetal bovine serum (7). These cells were then injected intraperitoneally into pristane (2,6,10,14-tetramethylpentadecane)-treated mice. Ascitic fluid was harvested 10 to 21 days after tumor cell implantation and centrifuged at 5,000 × *g* for 5 min to remove large debris. The resulting supernatant fluid was then centrifuged at 100,000 × *g* for 1 h. Finally, this supernatant was pooled, aliquoted, and stored at −70°C until use. This MAb, H7-2, which was reacted with TMEV VP-1 by Western (immuno-) blotting analysis, is an immunoglobulin G of the 2b subclass and has the ability to neutralize TMEV (7).

Antibody determination. Antibodies to TMEV in serum and cerebrospinal fluid were quantified by enzyme-linked immunosorbent assay (ELISA) performed as described by Rice and Fujinami (14). The TMEV antigen preparation was made as a cytoplasmic extract and processed as described by Talbot et al. (19).

Viral plaque assay. TMEV was quantified by plaque assay

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† This report is dedicated to the memory of our colleagues Peter W. Lampert and Alexander Rosenthal.

TABLE 1. Viral titers in the brains of individual mice at 1 month p.i.

Animal no.	Viral titer[a] at 1 mo p.i. in the brains of mice in:			
	Group 1 (treated *nu/nu*)	Group 2 (control *nu/nu*)	Group 3 (treated *nu/+*)	Group 4 (control *nu/+*)
1	350[b]	>1,000[b,c]	Undetectable[d]	Undetectable
2	125	>1,000[b,c]	Undetectable	Undetectable
3	25	>1,000[b,c]	Undetectable	Undetectable
4	15	>1,000[b,c]	Undetectable	Undetectable
5	4	150	Died[e]	Undetectable
6	4	60		
7	Undetectable	50		
8	Undetectable	Died[e]		
9	Undetectable	Died		
10	Undetectable	Died		
11	Undetectable	Died		
12	Undetectable	Died		

[a] PFU/mg of tissue from uninoculated cerebral hemisphere. Data from individual mice.
[b] Mice sacrificed when paralyzed or moribund.
[c] Animals with clinical signs; PFUs are estimates since higher dilutions were not performed.
[d] Detection level was 2 PFU/mg of tissue.
[e] No tissue obtained.

TABLE 2. ELISA anti-TMEV titer at 1 month p.i.

Substance tested[a]	ELISA anti-TMEV titer in animals from:			
	Group 1 (treated *nu/nu*)	Group 2 (control *nu/nu*)	Group 3 (treated *nu/+*)	Group 4 (control *nu/+*)
Serum	1/25,600	—[b]	1/102,400	1/6,400
	1/102,400	—	1/102,400	1/25,600
	1/102,400	—	1/102,400	1/25,600
	1/102,400	—	Died	1/25,600
Cerebrospinal fluid	1/80	—	1/320	1/320
	1/320	—	1/1,280	1/320
	1/320	—	1/1,280	1/5,120
	1/1,280	—	Died	1/5,120

[a] Four animals were chosen at random from each group to provide serum and cerebrospinal fluid samples.
[b] —, Undetectable.

on BHK-21 cells. Dilutions of material to be assayed (100 μl) were added to 35-mm plates (Costar, Cambridge, Mass.). The virus was allowed to adsorb for 1.5 h at room temperature, after which cell monolayers were overlaid with agarose. The overlay medium consisted of medium 199, 2% fetal bovine serum, and 0.5% agarose. After 3 days at 37°C, cell monolayers were fixed with 10% formaldehyde, stained with crystal violet, and enumerated for viral plaques.

Virus neutralization. The ascitic fluid containing anti-TMEV MAb was serially diluted in phosphate-buffered saline (PBS). The various dilutions were incubated at 4°C with approximately 200 PFU of TMEV for 1 h. The virus-antibody mixtures were then added to confluent BHK-21 monolayers and incubated for 1 h at 37°C. These monolayers were overlaid with agarose, and the monolayers were processed as described above. A dilution resulting in 50% reduction in PFUs was considered the endpoint dilution.

Mice and MAb treatment. Twenty-four homozygous athymic (*nu/nu*) and thirteen heterozygous (*nu/+*) nude mice

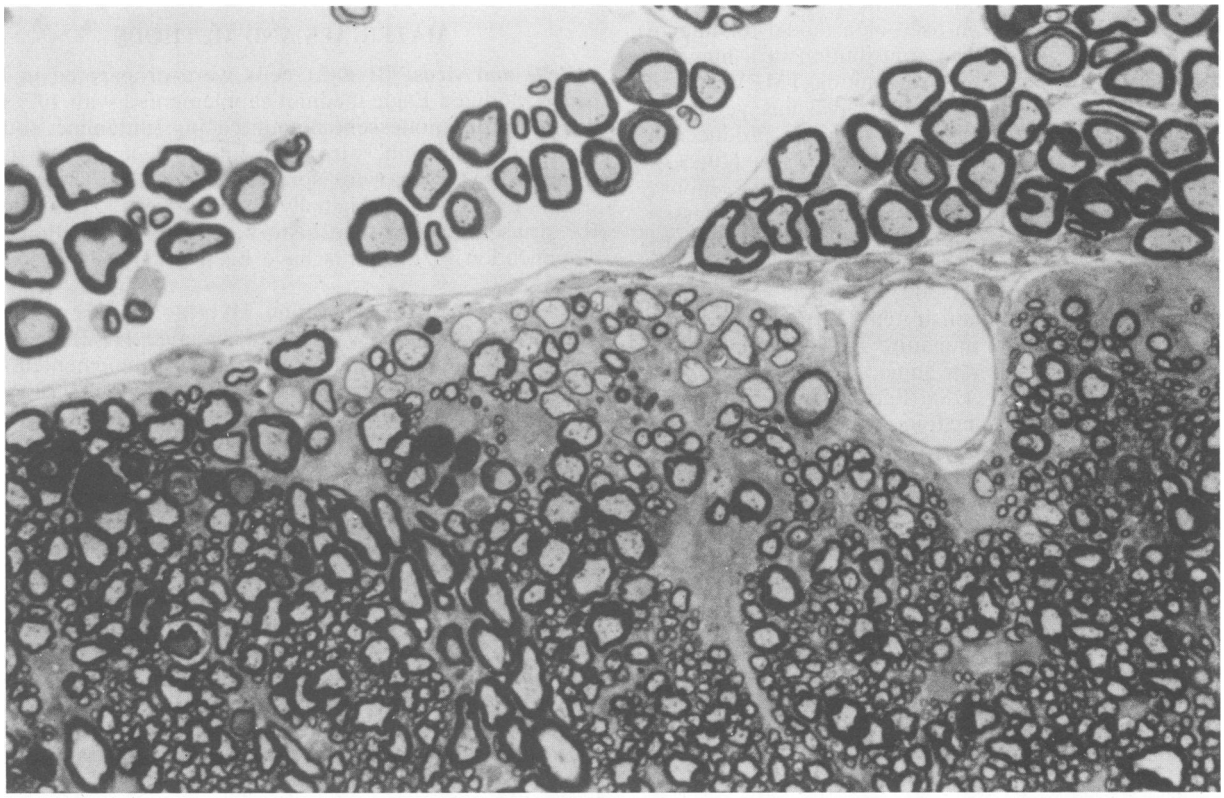

FIG. 1. Semithin section stained with toluidine blue (18). Small subpial plaque in the vicinity of the anterior root entry zone of an MAb-treated *nu/nu* mouse. Beginning of remyelination is seen in most of the involved axons at 1 month p.i. Approximate magnification, ×400.

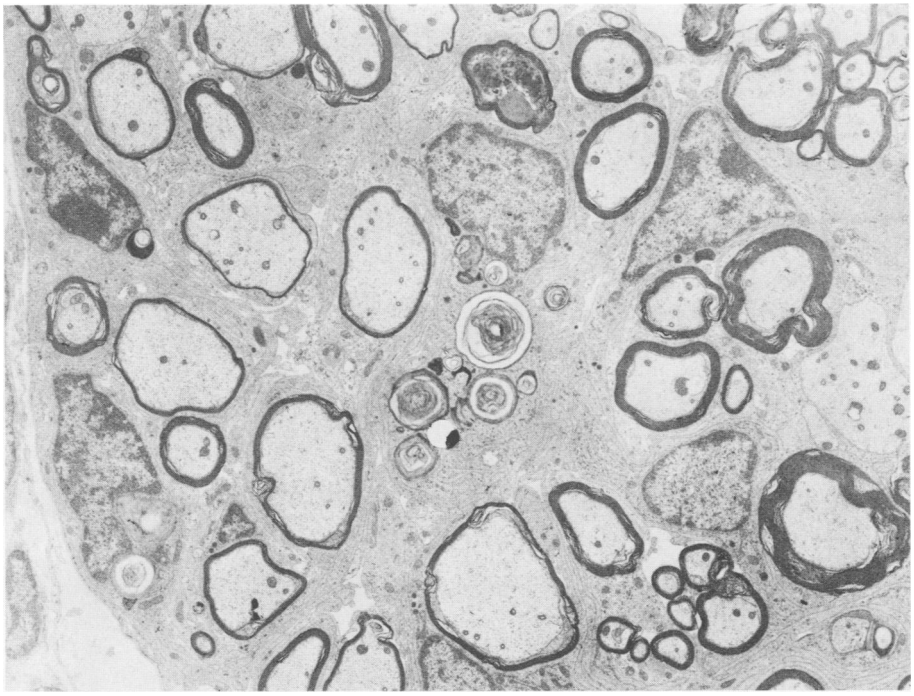

FIG. 2. Electron microscopy of region shown in Fig. 1 suggests remyelination. Thinly myelinating (remyelinated) axons and myelin debris are evident at 1 month p.i. Approximate magnification, ×1,500.

(Athymic Mouse Facility Cancer Center, University of California at San Diego, School of Medicine, La Jolla, Calif.) were inoculated in the left cerebral hemisphere with 5×10^5 PFU of TMEV (Daniels strain) at 4 weeks of age.

Mice were divided into four groups. Twelve infected *nu/nu* mice (group 1) and five infected heterozygous *nu/+* mice (group 3) received anti-TMEV MAb H7-2 as follows. Stock ascitic fluid (0.2 ml; 11.5 mg/ml of immunoglobulin G) prepared as described above was diluted 1:2 in PBS and injected intraperitoneally on days 2, 4, 6, 8, 10, 13, 16, 19, 23, 27, and 31 postinfection (p.i.). This MAb had an ELISA titer of 20 (log 2) and a neutralization endpoint titer of 2.5×10^5.

Twelve infected *nu/nu* mice (group 2) and eight infected *nu/+* mice (group 4) were left untreated and served as controls. Two days after the last treatment (33 days, p.i.), all animals except three of the mice from group 4 were sacrificed and tissues were processed as described below. These remaining mice from group 4 were sacrificed at 3 months p.i. for morphological studies only.

Tissue and histology. Four animals from each of the four groups designated above were anesthetized with intraperitoneal administration of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, Ill.) and exsanguinated. The blood was collected and centrifuged, and the serum was harvested and stored at −70°C. Approximately 5 μl of cerebrospinal fluid was obtained from a puncture into the subarachnoid space. In all cases, clear fluid with no blood contamination was obtained, diluted to 20 μl in PBS, and frozen at −70°C. The brains from these animals were aseptically removed. The right (uninoculated) hemisphere was isolated, weighed, and frozen to −70°C in 2 ml of PBS. The remaining animals in each group were processed for virological and morphological studies as follows. Mice were anesthetized with intraperitoneal pentobarbital sodium, and the right cerebral hemisphere was removed and processed as described above. Animals were then perfused through the left ventricle with 4% glutaraldehyde in 0.01 M PBS. The spinal cords were removed and postfixed for 2 h. Representative blocks from all spinal cord levels were processed for plastic embedding as previously described (18).

RESULTS

Survival and clinical signs of treatment. To assess the contribution of the neutralizing MAb H7-2 on survival of mice after TMEV infection, athymic *nu/nu* mice were infected intracerebrally with Daniels virus and MAb treatment was initiated on day 2 and boosted at the times listed in Materials and Methods. Control groups included MAb-treated heterozygous *nu/+* mice as well as untreated *nu/nu* and *nu/+* mice. MAb-treated infected homozygous *nu/nu* mice (group 1) showed no mortality by 4 weeks p.i. Of 12 animals in this group, 11 were active and virtually free of neurologic signs. However, the twelfth (animal 1 of group 1) became emaciated and suffered from paraplegia of the hind limbs, which was first noted at 29 days p.i.

In contrast, approximately half of the matched but untreated infected *nu/nu* mice (group 2) developed hind limb paralysis by 2 weeks p.i. These animals usually died within several days of showing such clinical signs. Only 3 of 12 animals in group 2 (animals 5 to 7) survived to 1 month p.i. All heterozygous *nu/+* mice in groups 3 and 4 survived except one animal, a *nu/+* mouse treated with MAb (animal 5 of group 3) which died 5 days p.i. of unknown causes. Otherwise, animals from these groups appeared to be clinically unaffected during the observation period.

Viral replication. To determine the extent of virus replication, homogenized CNS tissue from the uninoculated (right) cerebral hemisphere of each mouse was plaqued on BHK-21 cells for the detection of infectious virus (Table 1). No CNS samples from five animals in group 2 (animals 8 to 12) and one animal (animal 5) in group 3 were obtainable for assay.

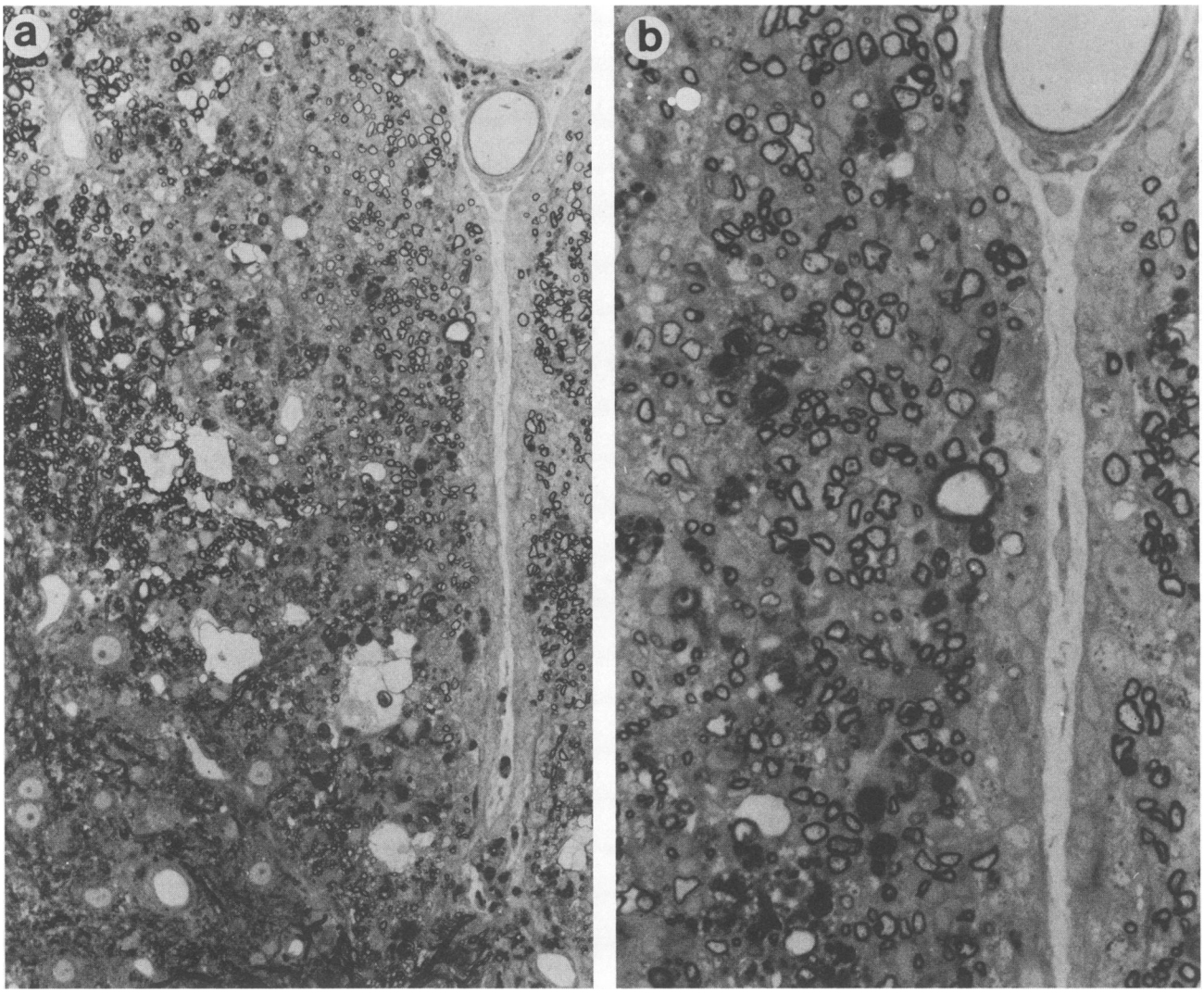

FIG. 3. Large plaque located in the anterior column of the spinal cord in a treated *nu/nu* mouse at 1 month p.i. Approximate magnifications, ×100 (a) and ×200 (b). Note completely denuded axons in the deeper white matter.

In addition, tissues from four animals in group 2 (untreated *nu/nu* mice 1 to 4) were harvested at various times prior to 1 month p.i. due to the development of neurologic signs and moribund condition by the mice. All other animals were sacrificed at 33 days p.i. except for three animals of group 4.

The results in groups 1 and 2 (Table 1) demonstrated that despite some variations among animals, *nu/nu* mice receiving MAb to VP-1 contained much less infectious virus as a group than did the untreated matched group (group 1 versus group 2). In fact, no virus was detectable (less than 2 PFU per mg of tissue) in 50% of animals from group 1. Interestingly, the one *nu/nu* mouse in the treated group (animal 1 of group 1) manifesting signs of paraplegia had the highest titer (350 PFU per mg of tissue).

By contrast, in the brains of heterozygous (*nu/+*) mice of both groups, no infectious virus was evident by plaque assay at 1 month p.i. (groups 3 and 4). However, previous studies (18) on heterozygous animals indicated that spinal cords often have low titers of infectious virus when none can be isolated from the brain (unpublished observation).

Quantitation of TMEV antibody by ELISA (Table 2) showed that infected, untreated *nu/nu* mice (group 2) had no detectable TMEV antibody in the cerebrospinal fluid or serum at 1 month p.i. Levels of antibody to TMEV in the serum of MAb-treated infected *nu/nu* and *nu/+* mice were as high and in most instances higher than those observed in untreated infected *nu/+* mice. However, anti-TMEV activity in the cerebrospinal fluid as measured by ELISA was slightly higher in untreated immunocompetent *nu/+* mice than in treated *nu/nu* mice and was comparable with that in the treated *nu/+* mice.

Altered morphologic pattern of disease. The natural history of the TMEV infection in *nu/nu* mice (17, 18) has been described. In the present study, control untreated *nu/nu* mice (group 2) examined at approximately 1 month p.i. manifested both white and gray matter abnormalities and support these previous studies. Occasionally, anterior horn cells showed neuronal vacuolar degeneration without accompanying formation of microglial nodules.

In white matter, plaques consisting of demyelinated axons

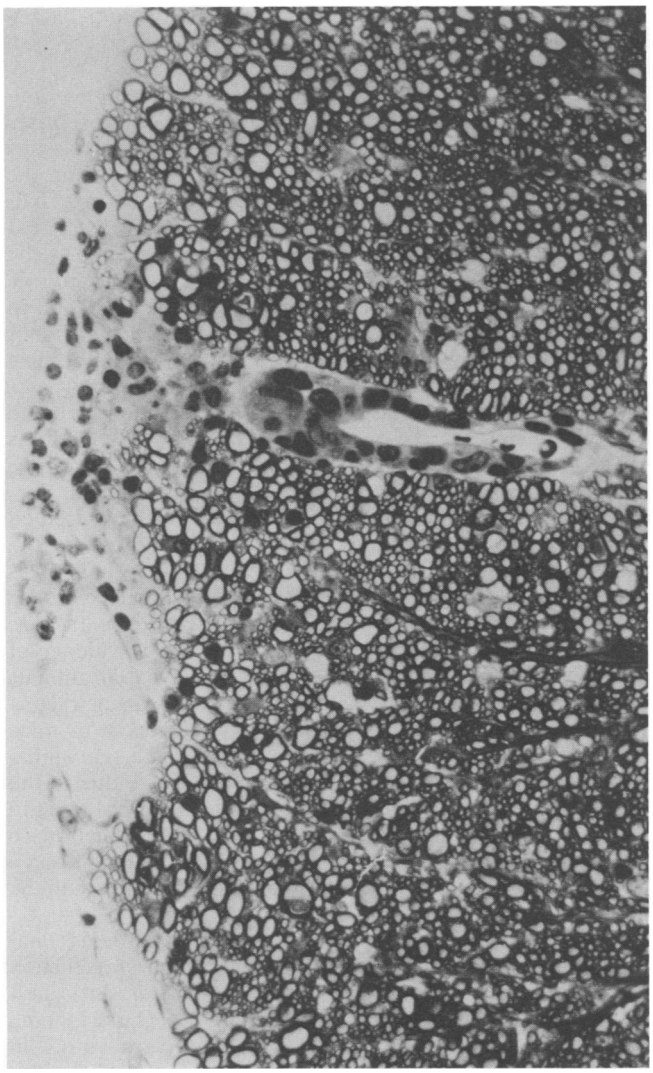

FIG. 4. Meningeal and perivascular inflammation in a TMEV-infected heterozygous (*nu*/+) mouse at 1 month p.i. Only occasional demyelinated axons are seen in association with the cellular infiltrates. Approximate magnification, ×100.

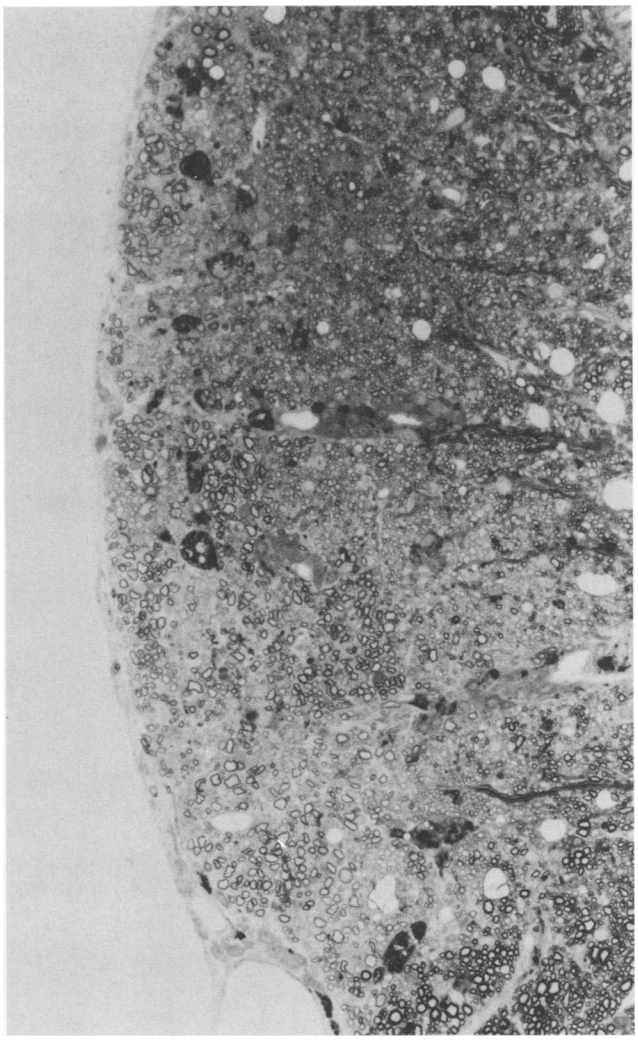

FIG. 5. Large inflammatory lesion in a TMEV-infected *nu*/+ mouse at 3 months p.i. The majority of involved axons are significant for thin, compacted loops of myelin. Approximate magnification, ×100.

in association with spongiform changes of the myelin were noted. Numerous necrotic glial cells could be found in the involved areas. Macrophages containing digested myelin debris were seen at the peripheries of zones with demyelinated axons. However, the meninges and perivascular areas developed only scanty inflammatory infiltrates. Electron microscopy identified these few infiltrating cells as macrophages and mononuclear cells. Little or no evidence of remyelination was noted by light or electron microscopy in these control animals (group 2).

In half (4 of 8) of the treated *nu*/*nu* animals examined concurrently with group 2 (animals 2 to 5), lesions of various sizes were noted. In contrast to the untreated controls, widespread remyelination was noted in most of the plaques in the anti-TMEV MAb-treated *nu*/*nu* group at 1 month p.i. (group 1). Small lesions developing in the subpial region underwent initial remyelination in almost all affected axons (Fig. 1 and 2). Larger demyelinating lesions of the anterior and lateral columns characteristically displayed extensive remyelination (Fig. 3a and b). Denuded axons were more likely to be found deeper in the involved white matter. The other four animals (animals 7 to 10) were clinically normal and had little or no morphologic evidence of spinal cord involvement. Neither spongiform myelinopathy nor necrosis of the gray matter was observed in treated *nu*/*nu* mice (group 1), and astroglial proliferation was minimal in both groups 1 and 2.

Infected immunocompetent *nu*/+ animals from both MAb-treated and untreated animals (groups 3 [four mice] and 4 [five mice]) had similar pathologic changes at 1 month p.i. These lesions consisted of focal subpial (Fig. 4) and, to a lesser extent, perivascular infiltrates, usually in the anterior and lateral columns. Meningeal inflammation also extended into the Virchow-Robin spaces of the white matter. Associated with these collections of inflammatory cells were small groups of naked axons. Macrophages were occasionally observed to be stripping myelin sheaths. Although the great majority of affected axons was completely demyelinated, occasional cylinders would show several loops of compacted myelin suggestive of early remyelination.

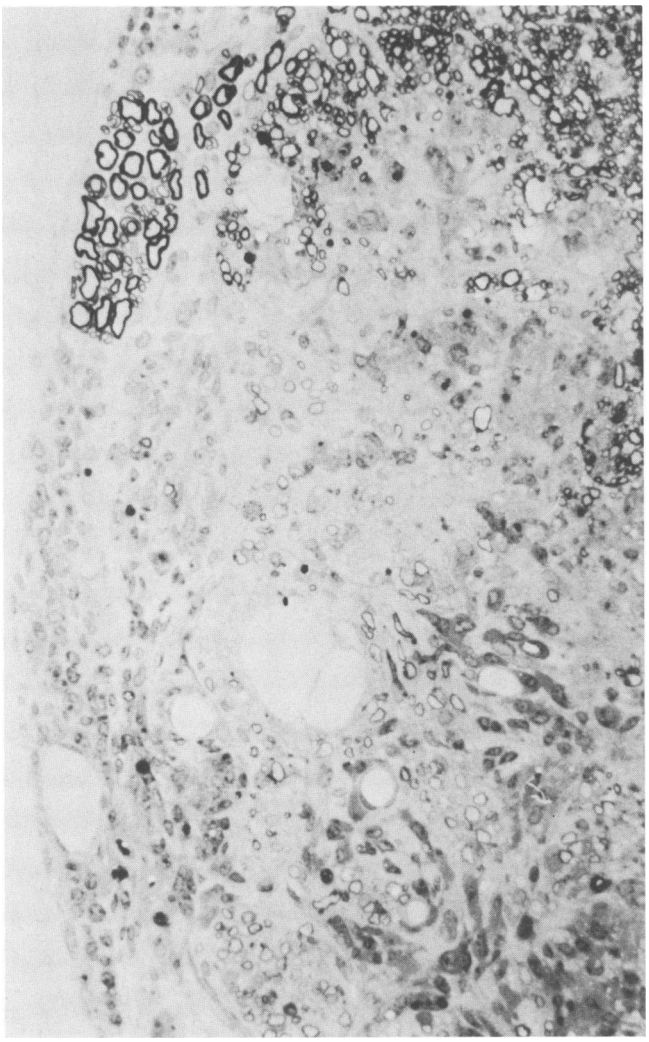

FIG. 6. Untreated *nu*/+ mouse at 3 months p.i. shows almost complete remyelination in a plaque located in the lateral column of the cervical spinal cord. Approximate magnification, ×100.

A group of three infected, untreated *nu*/+ mice from group 4 were monitored for 3 months p.i. and histologically examined. Large demyelinating lesions centered about the anterior root entry zone were commonly found and extended deeply into the cord (Fig. 5). Higher segments of the spinal cord were generally more severely affected. Most animals showed heavy inflammatory infiltrates intimately associated with the lesions. At this later time point, involved axons were well into the remyelinating process, which was visible as thin loops of myelin clearly observable under the light microscope (Fig. 6).

DISCUSSION

In this study, neutralizing anti-TMEV MAb to VP-1 passively transferred into TMEV-infected immunoincompetent *nu/nu* mice dramatically reduced amounts of infectious virus in brain, increased survival, and facilitated remyelination compared with their untreated counterparts. Infected untreated *nu/nu* mice (group 2) developed plaques of demyelinated axons early in the course of the disease (7 to 10 days p.i.) without significant remyelination (18). Moreover, the untreated *nu/nu* mice failed to produce any anti-TMEV antibody, as measured by ELISAs of their sera and cerebrospinal fluid.

Recent experiments have shown that these athymic animals do not mount detectable T-cell proliferative responses to viral antigens (unpublished data). Viral plaque assays demonstrated large quantities of infectious virus within the CNS. The TMEV titer at about 4 weeks p.i. was approximately 10^4 times greater than that found in infected SJL/J mice studied at the same time point (18). Whether it is the lack of a humoral response (known to play an important role in the control of picornavirus infections [21]) or a deficit in cellular immunity (6, 15, 20), *nu/nu* mice clearly cannot effectively halt the progression of this viral infection. The sum of these morphologic and immunologic data suggests that in the presence of large amounts of infectious virus the CNS is hindered from regenerating myelin sheaths. It is possible that oligodendrocytes are not destroyed but are reversibly injured.

Morphologic comparison at 33 days p.i. of MAb-treated versus untreated *nu/nu* mice inoculated with TMEV suggested a number of remarkable changes. First, a much less pronounced demyelinating disease of the spinal cord was seen in *nu/nu* mice receiving antibodies (group 1). In fact, 50% of the treated animals showed little or no evidence of white matter involvement. In those treated *nu/nu* animals with prevalent demyelination, remyelination was characteristically found in the plaques. Untreated infected *nu/nu* mice (group 2) in this experiment failed to show such remyelinative changes at similar time points. This may be due to the survival of treated animals or the reductions of viral titers in CNS by MAb treatment, thus allowing remyelination to occur, or both. Antibody titers could be achieved in the CSF of treated *nu/nu* mice, presumably because of a breach in the blood-brain barrier.

In the *nu*/+ animals (group 4), the plaques of demyelination in their spinal cords showed areas of thinly remyelinated axons late in the first month p.i., although significant remyelination was not observed until 3 months p.i. The beginning of remyelination has also been reported by 21 days p.i. in TMEV-infected SJL/J mice, in which demyelinated axons first appeared at 15 days p.i. (3, 4). These data suggest that this difference in remyelinative capacity may not be due simply to prolonged survival of the animals. Mechanisms described by Rodriguez and colleagues (16), such as serum factors initiating remyelination, have not been ruled out.

We conclude that the passively transferred anti-TMEV MAb entered the CNS and greatly reduced infectious virus titers in *nu/nu* mice, which fostered remyelination. This did not occur in the absence of the MAb. A comparison can be made with *nu*/+ animals, which normally produce antiviral antibodies in response to infection. *nu*/+ mice showed similar low intra-CNS virus levels and also remyelinated over a period of months. Thus, these results suggest that abatement of the injurious agent causing demyelination (i.e., infectious virus) results in and correlates directly with the capacity for remyelination.

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