JOURNAL OF VIROLOGY, Mar. 1986, p. 992–997
0022-538X/86/030992-06$02.00/0

Vol. 57, No. 3

Demyelinating Lesions Due to Theiler's Virus Are Associated with Ongoing Central Nervous System Infection

MARIO CHAMORRO,† CHRISTINE AUBERT, AND MICHEL BRAHIC*
Département de Virologie, Institut Pasteur, 75724 Paris Cedex 15, France

Received 8 October 1985/Accepted 21 November 1985

We used in situ hybridization and immunocytochemistry to look for a correlation between virus expression and white matter lesions during late demyelinating disease due to persistent Theiler's virus infection. We found the following. (i) Tissue lesions developed at the site of virus infection. This correlation was not explained by infection of lymphocytes and macrophages. (ii) Large differences in the extent of pathology existed between mice. The amount of inflammation paralleled the number of cells containing viral RNA or viral capsid antigens. (iii) C57BL/6 mice, which are resistant to demyelination, were able to eradicate the infection. Our results are strongly in favor of a mechanism of demyelination in which viral gene products play a central role.

Theiler's murine encephalomyelitis virus (TMEV), a picornavirus of mice, is responsible for a biphasic disease of the central nervous system (CNS) (8). The first phase, or early disease, is an acute polioencephalomyelitis which culminates 2 weeks after intracerebral inoculation and during which virus replicates actively in neurons (2). Survivors of early disease are persistently infected and develop a new neurological entity called late disease (8). The pathological changes of late disease are first detected 1 to 2 months after inoculation and evolve over a period of approximately 1 year. They consist of white matter inflammation with primary demyelination and are very reminiscent of the lesions observed in human multiple sclerosis (4). The severity of early and late diseases varies with the virus and the mouse strain. For example, tissue culture-adapted strains of TMEV, including the DA strain (DA-TMEV), cause typical late disease but no clinically apparent early disease. Among inbred mouse strains, the SJL/J mouse is extremely sensitive to late disease, whereas C57BL/6 animals are virtually resistant (10).

Late disease poses two main questions of pathogenesis. (i) What is the mechanism which allows the virus to persist in the face of a specific immune response? (ii) What is the mechanism of demyelination? Some time ago we observed that TMEV RNA persists in glial cells at the site of tissue lesions (2). More recently, we found that the virus life cycle in these cells is restricted at the level of RNA transcription (3). The majority of infected cells contain, on the average, 500 copies of viral genome and do not synthesize detectable levels of viral capsid antigens. Restricted viral replication may allow host glial cells to survive infection for an extended period, providing the virus with a shelter against immune defense mechanisms.

The mechanism of demyelination is still controversial. The tissue lesions of late disease are reminiscent of those of experimental allergic encephalitis, suggesting an autoimmune mechanism. However, immunosuppression experiments gave ambiguous results (9, 13), and attempts to transfer the disease passively with lymphocytes were negative (M. Chamorro and M. Brahic, unpublished data). On the other hand, the presence of viral RNA and, in a minority of infected cells, viral capsid antigens in glial cells, including oligodendrocytes (12), suggests a direct role for viral gene products.

The experiments presented in this article were aimed at distinguishing between these two mechanisms of demyelination. We reasoned that, if the presence of viral products is required for the development of lesions, a correlation should exist, both in space and time, between viral infection and formation of lesions. On the other hand, if demyelination is autoimmune, tissue lesions should evolve independently of virus replication.

In these studies, we took advantage of two techniques, in situ hybridization and immunocytochemistry, which allow the detection of viral nucleic acids and antigens in tissue sections. Longitudinal sections of spinal cords of infected animals were scanned by microscope. Pathological changes were quantitated by counting perivascular inflammatory infiltrates and viral expression by counting cells containing viral RNA or viral capsid antigens. We observed a strong correlation, both spatial and temporal, between virus expression and the development of tissue lesions. Furthermore, we observed that C57BL/6 mice, a strain resistant to demyelination, are able to eradicate TMEV from their CNS during the first month after intracerebral inoculation. Together, our results suggest strongly that tissue lesions evolve as a result of local viral infection.

MATERIALS AND METHODS

Virus, animals. DA-TMEV was given to us by R. Roos (University of Chicago) after adaptation to BHK cells. This strain was plaque purified three times in our laboratory with BHK cells.

Three- to four-week-old female SJL/J and C57BL/6 mice were purchased from Jackson Laboratory. Mice were inoculated intracranially with $\sim 10^4$ PFU of DA-TMEV in 40 μl of phosphate-buffered saline.

Mice were sacrificed and perfused with fixative as previously described (6). The fixative contained 2% paraformaldehyde, 0.01 M $NaIO_4$, 0.075 M L-lysine, and 0.037 M phosphate buffer (pH 7.5) (PLP). Perfused tissues were postfixed, dehydrated, and embedded in paraffin as previously described (6).

Infectivity assay. Infectivity titers in mouse CNS were measured as follows. Brain and spinal cord were removed aseptically and homogenized together in 20 ml of minimal

* Corresponding author.
† Present address: Department of Microbiology, University of California, San Francisco, CA 94143.

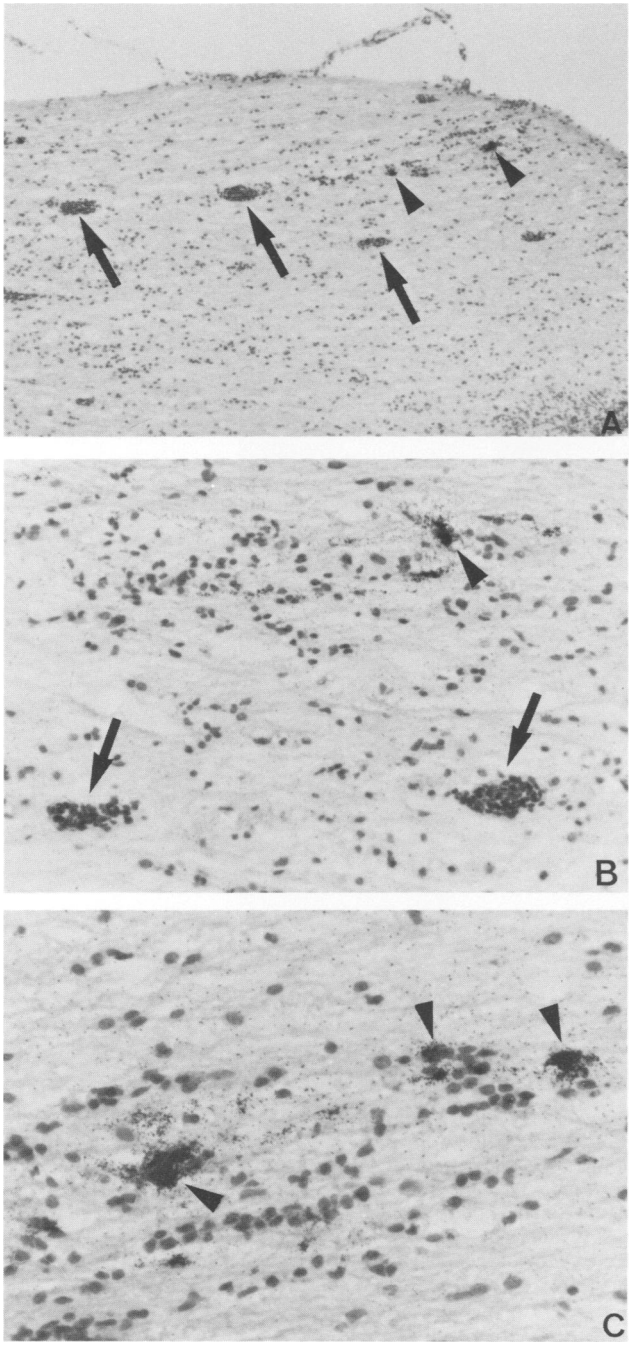

FIG. 1. Detection and quantitation of inflammatory tissue lesions and viral RNA in the spinal cord of an SJL/J mouse with late disease. The figure shows representative fields of longitudinal paraffin sections of spinal cord from an animal sacrificed 62 days after inoculation. The sections were hybridized in situ with TMEV-specific [^{3}H]cDNA (specific activity ~ 2×10^8 dpm/μg), exposed for 2 weeks, developed, and stained with hematoxylin-eosin. (A) A field observed at a final magnification of ×95. Arrows points to dense inflammatory foci, arrowheads to autoradiographic grain clusters. Both inflammatory foci and grain clusters were counted for the entire spinal cord to compute the data presented in Table 1. The contrast between black grain clusters and the histological stain is lost in these black and white pictures. (B) Final magnification, ×240. No viral RNA was observed in dense perivascular cuffs (arrows). Grain clusters were found instead in areas with diffuse inflammation (arrowhead). (C) Final magnification, ×385. The grain clusters (arrowheads) correspond to several adjacent cells containing viral RNA.

essential medium without serum. Homogenization was done at 0°C with a Polytron P-710 apparatus at a setting of 7. Four 30-s bursts were used for each sample. Homogenized tissues were clarified by centrifugation at 500 rpm for 5 min at 4°C. The supernatant, which was considered a 5×10^{-2} dilution, was serially diluted and assayed for infectivity on BHK cells by standard plaque assay procedures.

In situ hybridization. In situ hybridization on paraffin sections of mouse CNS was as previously described (6). [^{3}H]cDNA was prepared by reverse transcription of purified viral RNA as previously described (2). The specific activity of the cDNA was 2×10^8 dpm/μg. To count infected cells, longitudinal sections of the entire spinal cord were scanned by microscope at a final magnification of ×125. The majority (more than 90%) of infected cells contained ~500 copies of viral RNA (3). Since this corresponds to ~200 autoradiographic grains per cell after 2 weeks of exposure (1), these cells could be detected at low magnification.

Immunocytochemistry. The hyperimmune rabbit antiserum used has been described previously (1). This serum recognizes viral proteins VP0, VP1, and VP2 (data not shown). The serum was used at a 1/300 dilution. Immunoperoxidase staining was performed as previously described (1). Counterstaining was with hematoxylin. Antigen expression was quantitated by counting immunoperoxidase-positive cells at a final magnification of ×500. High magnification was required in this case, because the fraction of infected cells expressing viral antigens was small, making their detection difficult.

Simultaneous immunocytochemistry-in situ hybridization assay. The assay has been described in detail elsewhere (1).

RESULTS

Spatial distribution of tissue lesions and viral infection in white matter of the spinal cord. TMEV replication is restricted during late disease. The majority of infected cells contain low amounts of viral RNA and do not synthesize viral capsid antigens (3). Therefore, the only way to appreciate virus infection consists in detecting viral RNA with a highly sensitive in situ hybridization assay.

In the course of this study, we examined 24 SJL/J mice sacrificed between 2 and 4 months after intracranial inoculation. Several longitudinal sections of the entire spinal cord were examined for each animal. Inflammation was observed in 22 animals, with considerable variation of intensity from animal to animal. Spatial distribution of inflammation and viral RNA was appreciated best by systematically scanning the sections at a final magnification of ×312 after 2 weeks of autoradiographic exposure (Fig. 1B). Mononuclear cells were observed in two different configurations within inflammatory lesions: densely packed perivascular cuffs and diffuse infiltrates. Viral RNA was present in all lesions. It was never found in cuffs but was always present in areas of diffuse infiltration. In these areas, infected cells were often found in clusters (Fig. 1B and C). On occasion, viral RNA was found in isolated cells in normal-looking white matter.

Spatial association between viral RNA and inflammation can result either from recruitment of lymphoid cells at the site of parenchymal infection or from invasion of white

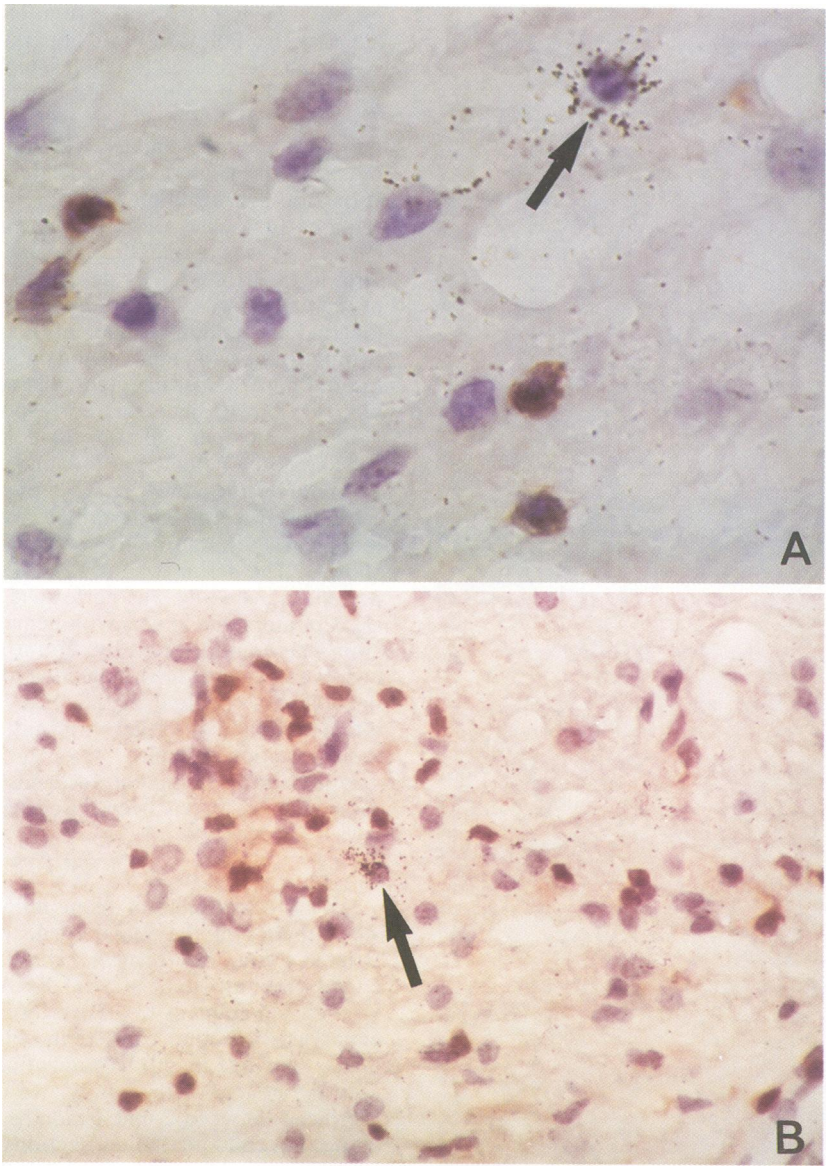

FIG. 2. Simultaneous in situ detection of viral RNA and inflammatory cell antigens. The figure shows representative fields of paraffin sections of the spinal cord of an SJL/J mouse sacrificed 62 days after inoculation. Antigens were detected in a first step with immunoperoxidase. In a second step, viral RNA was detected by in situ hybridization after acetylation of the slide to prevent nonspecific binding of radioactive cDNA probe (1). (A) Staining with antilymphocyte serum which was absorbed against uninfected SJL/J mouse brain extract. Inflammatory cells, lymphocytes, and macrophages stained brown. The arrow points to an infected cell not recognized by the serum. The slide was photographed at a final magnification of ×1,250. (B) Staining with anti F4/80 monoclonal antibody, a reagent specific for mouse macrophages (7). Macrophages stained brown. The arrow points to an infected cell not recognized by the serum. The slide was photographed at a final magnification of ×500.

matter by infected lymphocytes and macrophages. We distinguished between these possibilities with an assay for simultaneous in situ detection of viral RNA and inflammatory cell antigens (1).

A rabbit anti-mouse lymphocyte serum (Microbiological Associates) was absorbed exhaustively against uninfected SJL/J mouse brain homogenates. No immunoperoxidase staining was observed when reacting this serum with uninfected mouse CNS sections (data not shown). However, the absorbed serum stained lymphocytes and macrophages that were prepared from SJL/J mouse spleens, deposited on microscope slides, and fixed with PLP (data not shown). This absorbed serum was used for simultaneous detection of inflammatory cell antigens and viral RNA in sections of infected SJL/J mouse CNS. The majority of cells containing viral RNA were not stained by the serum (Fig. 2A); however, a small number of them were. Their frequency was measured by scanning longitudinal sections of spinal cord of two infected SJL/J mice. We found that ~10% of cells with viral RNA (22 of 303) stained with antilymphocyte serum. Because viral antigens have been observed in macrophages (5), we repeated this experiment using a monoclonal antibody specific for mouse macrophages (F4/80) (7). Figure 2B shows a representative field of late-disease lesions. A very large number of cells were stained by F4/80 monoclonal antibody, confirming that late-disease demyelinating lesions

TABLE 1. Quantitation of inflammation, viral RNA, and viral capsid antigen synthesis in the white matter of spinal cord at various times after intracerebral inoculation of SJL/J mice[a]

Animal no.	Time postinfection (days)	Viral RNA[b]	Viral antigens[c]	Inflammation[d]
1	7	0	0	0
2	7	0	0	0
3	14	0.5	0	0.65
4	14	0.07	0	0.26
5	21	0	0	0
6	21	0	0	0
7	30	0	0	0
8	30	0.1	0.06	0.44
9	61	3.5	1.1	2.9
10	61	2.1	0.4	2.3
11	62	0.74	0.25	1.7
12	62	11.0	5.2	5.5
13	62	3.1	1.5	6.9
14	62	12.7	ND	10.6
15	76	0	0	0
16	76	0	0	0
17	76	0.95	0.3	4
18	76	0.4	0.4	1.5
19	76	3.6	1.0	6.1
20	76	3.6	0.9	5.1
21	92	0.7	0.1	2.1
22	92	24.5	2.1	6.2
23	92	35	1.4	8.0
24	99	20.5	0.9	8.4
25	99	1.5	0	2.1
26	99	6.4	0	1.7
27	124	0	0	0
28	124	2.9	1.1	2.7

[a] Three-week-old SJL/J females were inoculated intracranially with 10^4 PFU of DA-TMEV. At the indicated times, animals were sacrificed and perfused with PLP fixative. Longitudinal paraffin sections of the entire spinal cord were used for detection of viral RNA by in situ hybridization, detection of viral capsid antigens by immunocytochemistry, and evaluation of pathology.

[b] After 2 weeks of autoradiographic exposure, the sections were scanned by microscope at a final magnification of ×125, and autoradiographic grain clusters were counted. The results are expressed as number of clusters per mm of spinal cord. Two longitudinal sections of each spinal cord were examined. The table presents the average of the two.

[c] The sections were scanned at a final magnification of ×500, and immunoperoxidase-positive cells were counted. The results are expressed as number of positive cells per mm of spinal cord. ND, Not done.

[d] Inflammation was quantitated by counting the number of perivascular cuffs observed during systematic scanning of slides used for in situ hybridization and immunoperoxidase. The results are expressed as number of cuffs per mm and are the average of three slides.

are very rich in macrophages. Only a small number of cells containing viral RNA were recognized by F4/80 monoclonal antibody. Their frequency was ~9%. In conclusion, a minority (10%) of cells containing viral RNA are inflammatory cells of macrophage type. Whether these cells replicate the virus or scavenge infected glial cells cannot be determined from our experiments.

In summary, this part of the study showed the existence of a strong spatial correlation between tissue lesions and the presence of infected cells. This correlation could not be explained by infection of inflammatory cells.

Temporal variation of virus infection and tissue lesions in the white matter of infected SJL/J mice. The experiments described above suggested the existence of a cause and effect relationship between virus infection and tissue lesions. If such is the case, the amount of tissue damage is expected to vary according to the amount of viral products expressed. We decided to look for a quantitative correlation between inflammation, viral RNA, and viral antigens at different times after inoculation. Quantitation of each parameter was obtained as follows. Two longitudinal sections of the entire spinal cord of each animal were hybridized in situ with TMEV [^{3}H]cDNA and exposed for 2 weeks; two more sections were stained for TMEV antigen with immunoperoxidase. Each hybridized section was scanned by microscope at a final magnification of ×125 (Fig. 1A). Inflammatory foci and autoradiographic grain clusters were counted. Fig. 1A shows a representative microscope field. Inflammatory foci are indicated with arrows and grain clusters with arrowheads.

Table 1 presents the results obtained with 28 SJL/J mice sacrificed between 7 and 124 days after intracerebral inoculation. Figures for viral RNA were significantly lower than the number of infected cells, because most infected cells occurred in groups containing up to 10 adjacent cells (Fig. 1C), and at low magnification these groups were counted as a single event. A wide range of viral RNA and antigen content was observed from animal to animal (e.g., viral RNA varied from 0 to 35 clusters per mm of spinal cord). Similarly, inflammation ranged from to 0 to 10 foci per mm of spinal cord. Strikingly, viral RNA, viral antigens and inflammation varied in parallel. No animal had inflammation without viral RNA.

Variations with time of infection were also remarkable. Some degree of inflammation and viral RNA was observed in white matter 2 weeks after inoculation, a time which corresponds to early disease. Inflammation in white matter at this stage had not been described in previous studies. Late disease began 1 to 2 months postinoculation. Wide variations of disease parameters were observed among animals sacrificed at a given time. Because all animals had lesions at 2 months, it is unlikely that this lack of pattern reflects variable time of onset for late disease. Instead, it suggests that the disease followed an unpredictable course in each animal.

Virus expression in animals resistant to demyelination. The severity of late disease varies among different inbred mouse strains; whereas the SJL/J strain is highly sensitive, C57BL/6 animals are practically resistant (10). This observation can be used to study host factors responsible for demyelination. In this context it was shown that SJL/J and C57BL/6 mice are equally susceptible to TMEV infection, a result interpreted as favoring an immunopathological, rather than a cytolytic, mechanism of demyelination (10). However, alternative interpretations are possible. For example, TMEV could persist in different cell types in C57BL/6 and SJL/J mice. Therefore, we decided to compare the locations and amounts of viral RNA and antigens in the CNS of C57BL/6 and SJL/J mice.

Three-week-old female C57BL/6 mice were inoculated with 10^4 PFU of DA-TMEV. Virus clone, dose, and inoculation route were identical to those used in the study of SJL/J animals reported above. Animals were sacrificed at various times postinfection (Table 2). Transverse sections of brain (forebrain, midbrain, and cerebellum) and longitudinal sections of spinal cord were processed as described above for SJL/J mice. In brain, viral RNA could be detected in low amounts 14 and 21 days postinoculation in neurons of cortex and hippocampus in 2 of 4 mice examined. Similar findings were obtained with SJL/J mice, showing that, in spite of the absence of clinically apparent early disease, some infection of neurons occurs with tissue culture-adapted TMEV.

The results obtained with spinal cord are summarized in

Table 2. Gray as well as white matter was scanned under the microscope at a final magnification of ×500 to locate viral RNA and antigens. Occasional positive cells were found up to 1 month postinoculation. Neither viral RNA and antigens nor inflammation was found after 1 month of infection. These results suggested that C57BL/6 mice are able to eradicate the virus from their CNS. Because persistent infection of the CNS of C57BL/6 mice had been described by others (10), we decided to compare the infectivity recovered by us from the CNS of SJL/J and C57BL/6 mice between 2 and 4 months postinoculation (Table 3). With one exception, infectivity could be measured in the CNS of SJL/J mice, although large differences in titer were observed from animal to animal. On the other hand, infectivity was recovered from only one C57BL/6 mouse of eight tested.

Taken together, our results suggest strongly that the resistance of C57BL/6 mice to virus-induced demyelination is linked to their resistance to TMEV infection. Therefore, the study of TMEV replication in C57BL/6 reinforces the notion that ongoing viral infection is a prerequisite for demyelination.

DISCUSSION

The mechanism of demyelination observed during late disease is still controversial. Two diametrically opposed mechanisms have been proposed. (i) Demyelination is an autoimmune process initially triggered by viral infection. (ii) Demyelination results from local virus infection. Our experiments were designed to distinguish between these alternatives. We showed the existence of a strong correlation between tissue lesions and viral infection. First, inflammatory lesions develop only in areas of white matter where a relatively large number of cells contain viral RNA. This spatial association does not result from the infection of lymphocytes or macrophages. Second, the extent of inflammation among SJL/J mice parallels the number of infected cells. Last, C57BL/6 mice are capable of clearing the infection and do not develop demyelination. Therefore, our results strongly favor a mechanism of demyelination in which viral gene products play a major role. Which role is still an open question. Viral antigens could induce an immune response directed at infected glial cells. The inflammatory aspect of the lesions speaks in favor of this. However, picornaviruses classically do not insert viral antigens at the cell surface, and capsid antigen expression is considerably restricted during late disease. Furthermore, since chronic treatment of infected mice with cyclophosphamide and antilymphocyte serum does not halt demyelination (13), it is unlikely that immune-mediated reactions account for all of the tissue damage. A direct effect of virus infection on myelin metabolism should be considered. This could be either lysis of the rare cells which accumulate high amounts of viral RNA and capsid antigens or alteration of the metabolism of persistently infected glial cells without cell killing (11).

This study revealed a previously unrecognized aspect of late disease. We observed very wide variations of disease intensity between animals sacrificed either at the same time or at different times after inoculation. Serial sectioning of the same tissue block showed that this was not a sampling artifact. Since the animals were inbred and had been inoculated with the same dose of plaque-purified virus, this variability strongly suggests that the disease follows an irregular, unpredictable course.

TABLE 2. Quantitation of inflammation, viral RNA, and viral capsid antigen synthesis in the spinal cord of C57BL/6 mice[a]

Animal no.	Time postinfection (days)	Viral RNA	Viral antigens	Inflammation
1	7	0	0	0
2	7	0	0	0
3	14	0	0	0
4	14	0.04	0	0.42
5	21	0	0	0
6	21	0.06	0	0.07
7	31	0	0	0
8	31	0.04	0	0.12
9	60	0	0	0
10	60	0	0	0
12	76	0	0	0
13	76	0	0	0
14	92	0	0	0
15	92	0	0	0

[a] Three-week-old female C57BL/6 mice were inoculated with 10^4 PFU of DA-TMEV and processed as described in Table 1. Values for viral RNA, viral antigens, and inflammation are as described in Table 1.

TABLE 3. Recovery of infectivity from the CNS of SJL/J and C57BL/6 mice inoculated intracerebrally with DA-TMEV[a]

Time postinoculation (days)	No. of PFU/CNS	
	SJL/J	C57BL/6
62	1.3×10^1	$<1.3 \times 10^1$
62		$<1.3 \times 10^1$
62		$<1.3 \times 10^1$
62		$<1.3 \times 10^1$
76	$<1.3 \times 10^1$	$<1.3 \times 10^1$
76	2×10^1	5.3×10^1
76	2×10^2	
76	3×10^2	
76	3×10^3	
76	6.8×10^3	
92	2.6×10^2	
92	1.9×10^3	
92	7.5×10^3	
92	6×10^4	
122	5×10^3	$<1.3 \times 10^1$
122	1.3×10^5	$<1.3 \times 10^1$

[a] For each mouse, the brain and spinal cord were removed, homogenized in 2 ml of minimal essential medium without serum, clarified, and assayed for infectivity. The results are given as PFU per 20 ml of original brain suspension.

ACKNOWLEDGMENTS

We thank Peter Ventura for excellent technical assistance, Evelyne Cash, Simona Ozden, and Frederic Tangy for many helpful discussions and M. C. Lupart for her patience with the preparation of the manuscript. A gift of F4/80 monoclonal antibody by S. Gordon is gratefully acknowledged.

This work was supported by grants from the National Multiple Sclerosis Society, the Fondation pour la Recherche Médicale, the Institut National de la Santé et de la Recherche Médicale, and the Institut Pasteur.

LITERATURE CITED

1. **Brahic, M., A. T. Haase, and E. Cash.** 1984. Simultaneous in situ detection of viral RNA and antigens. Proc. Natl. Acad. Sci.

USA **81**:5445–5448.
2. **Brahic, M., W. G. Stroop, and J. R. Baringer.** 1981. Theiler's virus persists in glial cells during demyelination disease. Cell **26**:123–128.
3. **Cash, E., M. Chamorro, and M. Brahic.** 1985. Theiler's virus RNA and protein synthesis in the central nervous system of demyelinating mice. Virology **144**:290–294.
4. **Dal Canto, M. C., and H. L. Lipton.** 1975. Primary demyelination in Theiler's virus infection: an ultrastructural study. Lab. Invest. **33**:626–637.
5. **Dal Canto, M. C., and H. L. Lipton.** 1982. Ultrastructural immunohistochemical localization of virus in acute and chronic demyelinating Theiler's virus infection. Am. J. Pathol. **106**:20–29.
6. **Haase, A. T., M. Brahic, L. Stowring, and H. Blum.** 1984. Detection of viral nucleic acids by in situ hybridization. Methods Virol. **7**:189–226.
7. **Hume, D. A., and S. Gordon.** 1983. Mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80. J. Exp. Med. **157**:1704–1709.
8. **Lipton, H. L.** 1975. Theiler's virus infection in mice: an unusual biphasic disease process leading to demyelination. Infect. Immun. **11**:1147–1155.
9. **Lipton, H. L., and M. C. Dal Canto.** 1976. Theiler's virus induced demyelination: prevention by immunosuppression. Science **192**:62–64.
10. **Lipton, H. L., and M. C. Dal Canto.** 1979. Susceptibility of inbred mice to chronic central nervous system infection by Theiler's murine encephalomyelitis virus. Infect. Immun. **26**:369–374.
11. **Oldstone, M. B. A.** 1984. Virus can alter cell function without causing cell pathology: disordered function leads to imbalance of homeostasis and disease, p. 269–276. *In* A. L. Notkins and M. B. A. Oldstone (ed.), Concepts in viral pathogenesis. Springer-Verlag, New York.
12. **Rodriguez, M., J. L. Leibowitz, and P. W. Lampert.** 1983. Persistent infection of oligodendrocytes in Theiler's virus-induced encephalomyelitis. Ann. Neurol. **13**:426–433.
13. **Roos, R. P., S. Firestone, R. Wollmann, D. Variakojis, and B. G. W. Arnason.** 1982. The effects of short-term and chronic immunosuppression on Theiler's virus demyelination. J. Neuroimmunol. **2**:223–234.