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LOCATION AND DISTRIBUTION OF VIRUS ANTIGEN IN THE CENTRAL NERVOUS SYSTEM OF MICE PERSISTENTLY INFECTED WITH THEILER'S VIRUS

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Summary.—The present study has shown that virus can be readily detected by immunofluorescent staining in the central nervous system (CNS) of SJL mice persistently infected with Theiler's murine encephalomyelitis virus (TMEV). Considering the low CNS virus content, large amounts of virus antigen were found in the white matter, the site of demyelinating lesions. Virus antigen was detected in all animals killed after post-infection (PI) Day 21, a time which can be considered as the beginning of the persistent phase of this infection, and the appearance of virus antigen in white matter corresponded closely in time with the onset of demyelination.

The pathogensis of this persistent infection can now be reasonably well reconstructed from the temporal observations made in this study. It would appear that between the second and third week PI, virus replication largely shifts from neurons in spinal cord gray matter to other cell types located in white matter. While a lower-grade persistent infection (in terms of the relative number of cells containing virus antigen) is established and maintained in cells in the gray matter and inflammatory and leptomeningeal infiltrates, cells in white matter appear to be mainly responsible for perpetuating the infection. Why these cells should supplant neurons as the most susceptible host cell during the chronic phase of the infection is discussed.

THE THEILER'S MURINE ENCEPHALOMYELITIS viruses (TMEV) are enteric pathogens of mice and members of the picornavirus family. Certain isolates, referred to as Theiler's original or TO strains, produce a unique biphasic central nervous system (CNS) disease in the natural host (Lipton, 1975; Lehrich, Arnason and Hochberg, 1976). Mice develop poliomyelitis during the first month after intracerebral inoculation, and in surviving animals an inflammatory demyelinating disorder occurs weeks to months later. It is of interest that myelin breakdown appears to be immune-mediated (Lipton and Dal Canto, 1976; Roos *et al.*, 1982).

At the time of poliomyelitis there is logarithmic growth of virus in the CNS, and virus antigen and RNA have been identified in the cytoplasm of neurons in gray matter (Lipton, 1975; Brahic, Stroop and Baringer, 1981). Thereafter, low levels of infectious virus can be recovered from the CNS, but cells containing virions have not been identified by routine electron microscopy (Dal Canto and Lipton, 1975). Only recently, in an ultrastructural immuno-histochemical study, has virus antigen been found in persistently infected mice (Dal Canto and Lipton, 1982). Virus antigen was observed primarily in macrophages in white matter, with lesser numbers of astrocytes, mononuclear inflammatory cells and terminal processes of neurones also containing virus antigen. In addition, Brahic *et al.* (1981) have

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recently reported that virus RNA was in glial cells in white matter of persistently infected mice by *in situ* hybridization. Thus this persistent infection is maintained by virus replication in a number of different host cells.

In the present study we have examined in detail the temporal development and distribution of virus antigen in the CNS of infected mice in an attempt to relate this to the pathogenesis of the demyelinating lesion. Virus antigen was readily detected in all persistently infected animals. Most of the virus antigen observed in the spinal cord was located in the white matter, appearing at roughly the time of onset of demyelination. To a much lesser extent, virus antigen was also detected in other locations, *i.e.*, in cells in leptomeningeal and perivascular infiltrates and in gray matter. Because virus antigen could be localized to the vicinity of demyelinating lesions, fluorescent-antibody-stained sections also were examined for immunoglobulins and complement (C3); however, no evidence was found of deposition of virus-antibody complexes in demyelinating lesions or elsewhere in the CNS.

METHODS

Virus.—Stock DA virus was prepared from the third suckling mouse brain passage as a clarified 10% suspension of brain in Eagle's minimum essential medium as described (Lipton, 1975).

Animals and animal inoculations.—Six-week-old, male SJL mice were obtained from Jackson Laboratory, Bar Harbour, Maine. Anaesthetized mice were inoculated in the right cerebral hemisphere with 0·02 ml containing approximately 1000 suckling mouse 50% mean lethal doses of virus (Lipton, 1975). Age-matched control mice were inoculated with an equivalent dilution of normal suckling mouse brain suspension or remained uninoculated.

Antisera.—Antiserum to GDVII virus was produced in young New Zealand rabbits given 4 i.v. inoculations of Cs_2SO_4 purified virus at 2-week intervals and then bled 2 weeks after the last injection. This antiserum, used at a dilution of 1:20–1:100, did not produce nonspecific staining of control mouse CNS tissues, and the reaction was blocked by prior incubation of sections containing virus with hyperimmune mouse serum. Goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) was purchased from Miles Laboratory (Elkart, IN) and used at a dilution of 1:20–1:50.

Heavy-chain specific goat antiserum to mouse IgG, IgM and IgA conjugated with FITC and goat antiserum to mouse C3 conjugated with FITC were purchased from Cappel Laboratories (Dowington, PA). Rabbit antiserum to mouse C3 conjugated with FITC was provided by M. B. A. Oldstone (LaJolla, CA). Both anti-mouse C3 sera stained C3 present in immune complexes in the glomeruli of lymphocytic choriomeningitis virus carrier mice provided by M. Popescu (Chicago, IL). Rabbit anti-human glial fibrillary acidic protein (GFAP) serum, provided by Lawrence Eng (Palo Alto, CA), was used to identify astrocytes.

Immunofluorescent antibody staining.—Anaesthetized mice were perfused with cold phosphate-buffered saline. The brains and spinal cords were removed, quick-frozen and sections (5–8 μm) were cut in a cryostat. One cm coronal spinal cord segments were placed in O.C.T. Compound (Lab-Tek Products) before freezing. The indirect technique for immunofluorescent staining was used for detection of virus antigen as previously described (Lipton, 1975) and GFAP, and the direct technique was used for staining immunoglobulins and C3. Fluorescent-antibody-stained sections were viewed with a Zeiss microscope with epifluorescence light excitation and the following filter system: excitation (440–490 nm), barrier (520 nm) and reflector (510 nm).

Histological strains.—Replicate frozen sections were alternately stained with rabbit anti-GDVII serum for fluorescent microscopy and with one of several histological stains for viewing by light microscopy. Sections were stained with haematoxylin and eosin or methyl green pyronine to delineate cellular detail, and for nonspecific esterase as described (Yam, Li and Crosby, 1971) to identify tissue macrophages. The Sudan IV stain for cholesterol and fat was used to delineate cells (mainly macrophages) that had phagocytozed myelin and other debris.

RESULTS

Location and relative amount of virus antigen in the CNS

Virus antigen was found primarily in the spinal cord and rarely in the brain of mice killed after PI Day 21, *i.e.*, during the persistent phase of the infection. Therefore, the spinal cord became the main focus of this study. Virus antigen was detected in the spinal cords of 15/16 mice killed between Days 6 and 164 (Table). A total of 52 of 58 spinal cord segments from

TABLE.—*Analysis of material for immunofluorescence staining from DA virus-infected SJL mice*

Animal number	Day after infection	Clinical observation	No. segments positive[a] / Total examined
0032	6	Well	0/4
1449	10	FP[b]	3/3
1452	16	FP	4/5
607	23	FP	3/4
457	26	FP	3/3
1071	32	FP	3/3
0007	41	FP	4/4
608	44	FP	2/3
1072	53	SP[c]	5/5
0006	56	SP	3/3
456	62	SP	2/3
464	90	Well[d]	1/3
1586	103	SP	6/6
1466	116	SP	4/4
465	118	SP	4/4
610	164	SP	5/5

[a] Spinal cord segments were recorded as positive if any of 10 coronal sections examined contained virus antigen (see text).
[b] Flaccid paralysis.
[c] Spastic paralysis.
[d] This animal had recovered from a slight gait abnormality that developed on PI Day 42.

these animals were found to contain antigen, and at least one section (but usually many sections) from these segments were positive. Of those mice killed after PI Day 21, 45 of 50 spinal cord segments were positive for antigen, and virus antigen could still be detected at the latest times studied (PI Days 90–164). Thus, in contrast to previous findings in outbred mice (Lipton, 1975), TMEV was readily detected in the CNS of SJL mice during the persistent phase of the infection.

Numerous sections of normal mouse CNS did not stain with the rabbit antiserum to GDVII virus and the second antibody, nor did sections from infected mouse CNS stain with normal rabbit serum and the second antibody.

Acute phase of the infection (PI Days 6–16)

A detailed immunofluorescence study of virus replication during the poliomyelitis phase in outbred mice has been reported (Lipton, 1975). For this reason, few infected mice were examined at early time-points. Virus antigen was first observed in the spinal cord on PI Day 10, at which time antigen-containing cells were confined to the gray matter. Virus antigen was identified primarily in large, pyramid-shaped cells bearing the appearance of neurons, and in smaller cells which could be neurons or astrocytes; however, in contrast to later times (see below) spinal cord sections showed at most only a few positive cells in any section. On PI Day 16, an occasional cell in the white matter as well as cells in gray matter were found to contain virus antigen. This is roughly the time when demyelination begins in this model (Dal Canto & Lipton, 1975). On PI Day 16, the antigen-containing cells in white matter were near the junction with the gray matter, suggesting that the infection had spread from nearby cells in the gray matter. These findings are consistent with an early phase of virus replication that takes place primarily in neurons, as previously suggested (Lipton, 1975; Brahic *et al.*, 1981).

Chronic phase of the infection (PI days 23–164)

Substantially more virus antigen was observed in the CNS after PI Day 21. At this time, virus antigen was detected in 3 locations: (i) the majority of cells laden

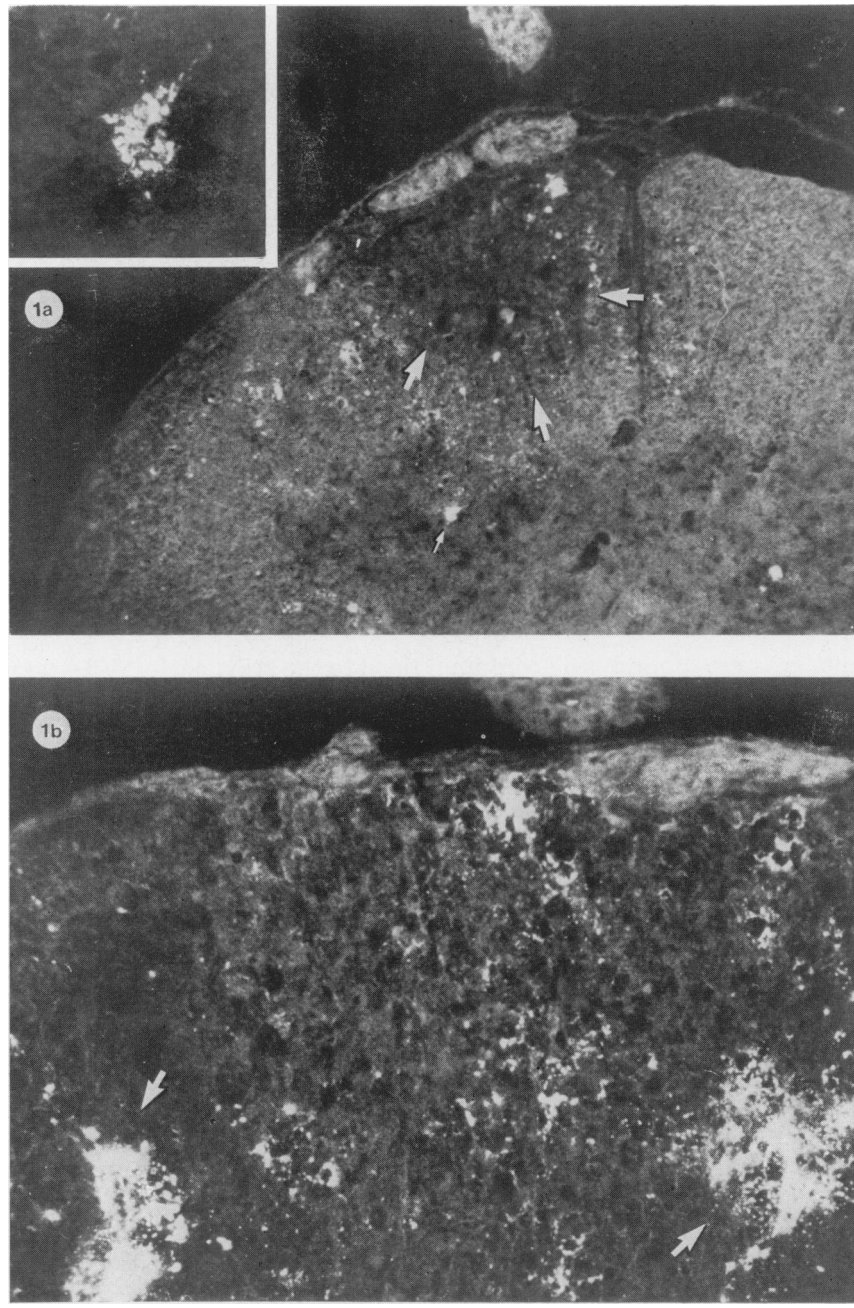


FIG. 1.—(a) Immunofluorescent-antibody-stained spinal cord section showing the relation of virus antigen to a severely demyelinated area (triangular darker counterstained area in the lateral coulmn delineated by large arrows and extending to the leptomeninges). PI Day 62. × 14. The insert shows virus antigen in a cell in the anterior horn region which may be a neuron. This cell is delineated by a small arrow on (a), × 90. (b) Immunofluorescent-antibody-stained spinal cord section showing area of white matter heavily infected with virus. Note the two clusters consisting of many cells laden with virus antigen (arrows). PI Day 41. × 58.

with antigen were present in white matter (1a and 1b). (ii) Many fewer cells containing antigen were observed in the anterior and posterior horns of the gray matter (Fig. 1a). Some of the larger cells in the gray matter staining for virus antigen were pyramidal in shape and were probably neurons, as in the acute phase of the infection (Fig. 1 insert). (iii) Finally, only occasionally were cells staining for virus antigen detected in inflammatory infiltrates in the leptomeninges and perivascular spaces (Fig. 2). The majority of these antigen-containing cells appeared to be mononuclear cells.

While virus antigen-containing cells in

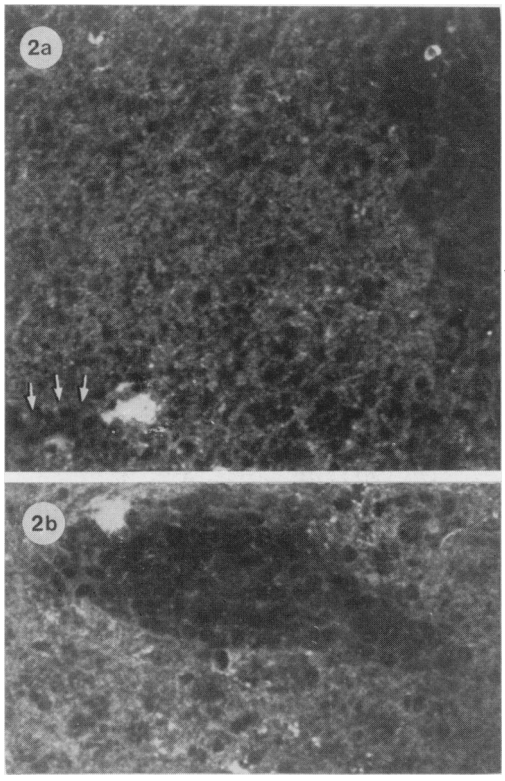


FIG. 2.—(a) Immunofluorescent-antibody-stained spinal cord section showing virus antigen in a single inflammatory cell in the leptomeninges (top right) and a larger cell at the margin of a perivascular cuff which is delineated by small arrows. PI Day 41. × 64. (b) Another section showing a cell in a perivascular cuff of mononuclear cells which contains virus antigen (the nucleus of this cell is out of the plane). × 64.

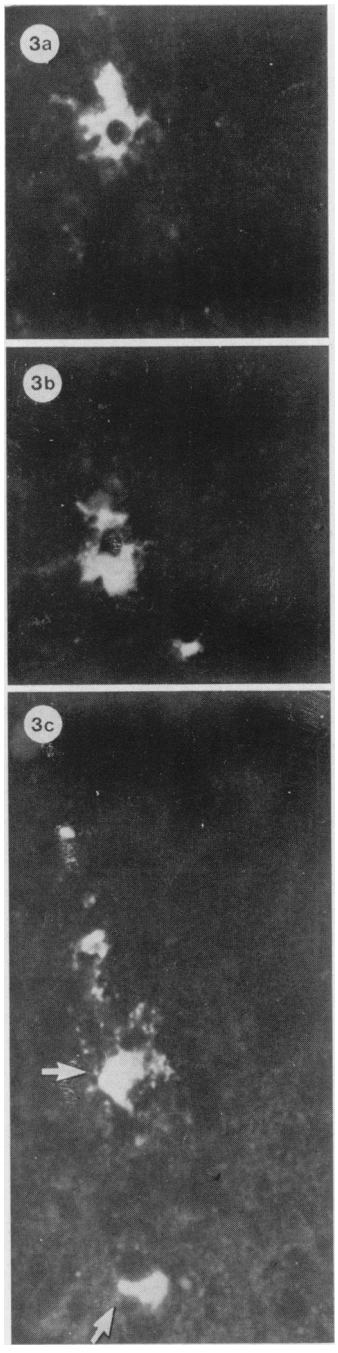
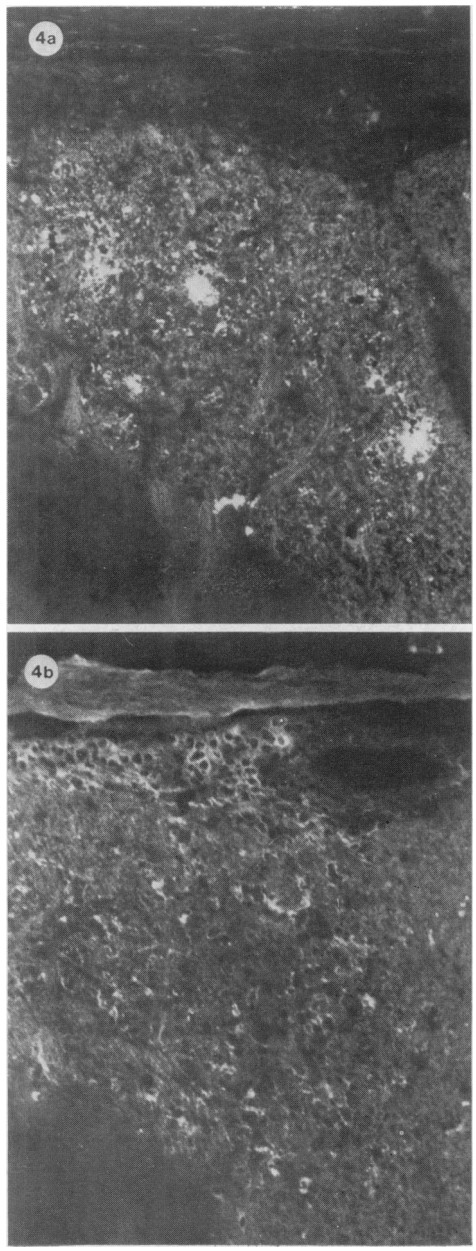


FIG. 3.—Immunofluorescent-antibody-stained sections of spinal cord from persistently infected mice showing 2 very large, irregular cells 50–100 nm in size (a and b) and several other very large cells (c) within the white matter. × 90.

white matter tended to be randomly distributed throughout the parenchyma between PI Days 23–32, such cells were located near the junction with the gray matter as described for PI Day 16. In terms of the amount of virus antigen present, few cells containing antigen were observed in any 6μm coronal section of the spinal cord; however, between PI Days 41 and 62 there was maximal involvement and numerous cells laden with antigen could be found in some areas (Fig. 1b). Also shown in Fig. 1b (and in 4a), are large clusters of cells with virus antigen which were often seen in the white metter. These clusters were composed of many cells, and while initially this appeared to be an artefact, exhaustive washing did not reduce the extent of the staining.



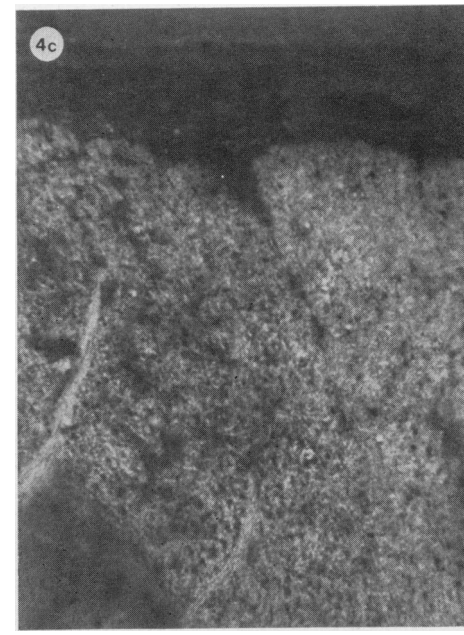


FIG. 4.—Closely spaced sections through an area of the posterior-column white matter of the spinal cord stained by the immunofluorescent antibody method for (a) virus antigen, (b) IgG and (c) C3. The posterior horn of the gray matter is in the lower left corner. PI Day 56. × 52.

While all segments of spinal cord white matter contained virus antigen, the thoracic ones were consistently most affected; virus antigen was also observed more frequently in the lateral than the anterior or posterior columns. The reason for this distribution is unclear; however, this is similar to the distribution of demyelinating lesions (Lipton, 1975; Dal Canto and Lipton 1975). Of interest was

the fact that virus antigen was frequently found to be aligned towards the margins of more severely demyelinated areas. The more darkly stained, wedge-shaped area adjacent to the root entry zone in Fig. 1a is typically severely demyelinated. Virus antigen was never observed in vascular endothelium or ependymal cells at any time during the infection.

Identification of the white matter cells containing virus antigen

The antigen-containing cells in white matter showed great variation in size and shape. Figure 3 shows virus antigen in a number of very large cells which because of their size (50–100 nm) and shape are possibly macrophages or reactive astrocytes. The identity of these cells could not be determined for certain from replicate frozen sections with one section stained with GFAP or nonspecific esterase, for it proved difficult to define an affected cell on both sections. On the other hand, it was possible to localize virus antigen to small circumscribed areas in the lateral columns where most of the cells on replicate section stained with the nonspecific esterase stain (not shown). Our experience from immunoperoxidase staining at an ultrastructural level has shown that most of the large cells in white matter containing the reaction product are indeed macrophages (Dal Canto and Lipton, 1982).

Failure to demonstrate deposition of virus–antibody complexes in the CNS

After detecting virus antigen in the CNS it was of particular interest to look for deposition of virus–antibody complexes in the same areas. As shown in Figs 4a and b, IgG was observed in areas in which virus antigen was demonstrated. The IgG was localized within the cytoplasm of cells in inflammatory infiltrates in both the leptomeninges and white matter. The cells staining for IgG were probably plasma cells since plasma cells have been demonstrated by electron microscopy in the leptomeninges, perivascular sites and demyelinating lesions in this infection (Dal Canto and Lipton, 1975), On the other hand, linear and granular deposits of IgG were not found in the CNS, nor was there stainable C3 in white matter lesions or elsewhere (Fig. 4c).

DISCUSSION

The present study has demonstrated that Theiler's virus can be regularly detected in the CNS of persistently infected SJL mice by immunofluorescence staining, and it has provided valuable information about the sites of virus replication. The discovery of relatively large amounts of virus antigen in the spinal cords of persistently infected SJL mice was unexpected, particularly in view of the low virus content in the CNS (Lipton, 1975; Lipton, Dal Canto and Rabinowitz, 1978) and our inability to detect virus by immunofluorescence staining in persistently infected outbred mice (Lipton, 1975). Although not well documented, the level of sensitivity of immunofluorescence staining in a productive infection is probably about 10^4 infectious particles per gram of tissue (El Mekki and van den Groen, 1981). This figure could vary depending upon the class of virus, and conceivably for chronic infections, by the mechanism of virus persistence. The CNS titres early in the chronic phase of TMEV infection (PI Days 21–90) approximate this level (Lipton, 1975; Lipton *et al.*, 1978). None the less, the large amounts of virus antigen demonstrated in the CNS of SJL mice seem disproportionate to the virus content. This could be explained if the assay system were inefficient, underestimating the titre, or if the immunofluorescence technique is more sensitive than realized.

The success of the present study in detecting virus by immunofluorescence staining may in part be due to the use of a more specific antiserum to purified TMEV (Lipton and Friedman, 1980) and higher CNS virus titres in SJL mice. Although the demyelinating disease in SJL mice is clearly more severe and evolves faster than

in other strains of mice, including outbred mice, we previously reported that CNS virus titres among a number of strains of mice were not substantially different during the persistent phase of the infection (Lipton and Dal Canto, 1979). Those comparisons were based on relatively few samples and a limited number of time-points; more extensive experience now leads us to believe that the virus content may be significantly higher in SJL mice (unpublished data).

We have shown that virus replication largely shifts from neurons in the spinal-cord gray matter to other cells located in white matter (Figs 1 and 2). While a lower-grade persistent infection (in terms of relative numbers of cells affected) is established and maintained in astrocytes, mononuclear inflammatory cells and possibly neurons, macrophages appear to be mainly responsible for perpetuating this infection (Fig. 3) (Dal Canto and Lipton, 1982). Why macrophages should supplant neurons as the most susceptible host cell is unknown. One possibility could be that not all neurons possess virus receptors, and those neurons with receptors are depleted during the acute phase of the infection. The virus would then infect the next most susceptible cell population, in this instance, primarily macrophages. Another possibility could be that since the shift in virus replication occurs after the host has mounted a neutralizing antibody response to virus (Rabinowitz and Lipton, 1976). macrophages by virtue of the Fc receptors bind and internalize infectious virus–IgG complexes. The ability of antibody to enhance virus growth in macrophages has been recently demonstrated for several classes of viruses (Kliks and Halstead, 1980; Peiris and Porterfield, 1979, 1981), and there has been speculation regarding this mechanism in virus persistence (Schlesinger and Brandriss, 1981). If the mononuclear inflammatory cells that contain TMEV turn out to be monocytes (Fig. 2), then virtually all of the virus antigen detected during the persistent phase of this infection could be accounted for by cells having Fc receptors. There is evidence that Fc receptors are on the surface of astrocytes (Traugott, Snyder and Raine, 1979), and oligodendrocytes may also bear Fc receptors (Ma *et al.*, 1981). While astrocytes are infected, oligodendrocytes have not been shown to contain virus antigen (Dal Canto and Lipton, 1982). A difference in susceptibility of cells bearing Fc receptors could be explained by differences in the number of Fc receptors or their affinity for IgG. The general mechanism of antibody facilitating virus entry into cells with Fc receptors would also explain how a persisting virus could evade the host's immune defences.

The association of TMEV antigen with demyelinating lesions (Fig. 1a) suggests that the virus is directly involved in the pathogenesis of myelin breakdown. Penney and Wolinsky (1979) reported finding virions in oligodendrocytes of mice infected with the WW strain of TMEV; WW virus forms intracytoplasmic inclusions, thereby permitting the detection of infected cells. Their model used suckling mice, but this does not necessarily mean that TMEV will replicate in oligodendrocytes in the CNS of a mature host, the consequence of which would be demyelination. In contrast, we have never found cytoplasmic inclusions in DA virus-infected adult mice, nor have virions been seen in any cell type (Dal Canto and Lipton, 1975). In addition, previous studies have supported an immune-mediated pathogenesis of demyelination (Lipton and Dal Canto, 1976; Dal Canto and Lipton, 1980; Roos *et al.*, 1982) The fact that TMEV now appears to primarily persist in macrophages, and the fact that reaction product to virus antigen has not been found in oligodendrocytes strongly support an immune mediated mechanism of demyelination (Dal Canto and Lipton, 1982). The exact way in which the host immune response produces demyelination remains to be elucidated; however, one possible mechanism, the deposition of immune complexes, has been excluded since antibody and complement were not

deposited in diseased areas of white matter that contained virus antigen (Fig. 4).

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REFERENCES

Brahic, M., Stroop, W. G. & Baringer, J. R. (1981) Theiler's Virus Persists in Glial Cells during Demyelinating Disease. *Cell*, **26**, 123.

Dal Canto, M. C. & Lipton, H. L. (1975) Primary Demyelination in Theiler's Virus Infection: An Ultrastructural Study. *Lab. Invest.*, **33**, 626.

Dal Canto, M. C. & Lipton, H. L. (1980) Schwann Cell Remyelination and Recurrent Demyelination in the Central Nervous System of Mice Infected with Attenuated Theiler's Virus. *Am. J. Path.*, **98**, 101.

Dal Canto, M. C. & Lipton, H. L. (1982) Ultrastructural Immunohistochemical Localization of Virus in Acute and Chronic Demyelinating Theiler's Virus Infection. *Am. J. Path.*, **106**, 20.

El Mekki, A. A. & van den Groen, G. (1981) A Comparison of Indirect Immunofluorescence and Electron Microscopy for the Diagnosis of Some Haemorrhagic Viruses in Cell Cultures. *J. virol. Methods*, **3**, 61.

Kliks, S. C. & Halstead, S. B. (1980) An Explanation for Enhanced Virus Production in Chick Embryo Cells. *Nature*, **285**, 504.

Lehrich, J. R., Arnason, B. G. H. & Hochberg, F. (1976) Demyelinative Myelopathy in Mice Induced by the DA virus. *J. neurol. Sci.*, **29**, 149.

Lipton, H. L. (1975) Theiler's Virus Infection in Mice: An Unusual Biphasic Disease Process Leading to Demyelination. *Infect. Immun.*, **11**, 1147.

Lipton, H. L. & Dal Canto, M. C. (1976) Theiler's Virus-Induced Demyelination: Prevention by Immunosuppression. *Science*, **192**, 62.

Lipton, H. L., Dal Canto, M. C. & Rabinowitz, S. C. (1978) In *Persistent Viruses: ICN–UCLA Symposia on Molecular and Cellular Biology*. Vol. XI, Ed. J. Stevens, G. J. Todaro and C. F. Fox. New York: Academic Press, p. 679.

Lipton, H. L. & Dal Canto, M. C. (1979) Susceptibility of Inbred Mice to Chronic Central Nervous System Infection by Theiler's Murine Encephalomyelitis Virus. *Infect. Immun.*, **26**, 369.

Lipton, H. L. & Friedman, A. (1980) Replication of GDVII and DA strains of Theiler's Murine Encephalomyelitis in BHK21 Cells: An Electron Microscopic Study. *Virology*, **101**, 389.

Lipton, H. L. (1980) Persistent Theiler's Murine Encephalomyelitis Virus Infection in Mice Depends on Plaque Size. *J. gen. Virol.*, **46**, 169.

Ma, B. I., Joseph, B. S., Walsh, M. J., Potvim, A. R. & Tourtellotte, W. W. (1981) Multiple Sclerosis Serum and Cerebrospinal Fluid Immunoglobulin Binding to Fc Receptors of Oligodendrocytes. *Ann. Neurol.*, **9**, 371.

Peiris, J. S. M. & Porterfield, J. S. (1979) Antibody-Mediated Enhancement of Flavivirus Replication in Macrophage Cell Lines. *Nature*, **282**, 509.

Peiris, J. S. M. & Porterfield, J. S. (1981) Antibody-Dependent Enhancement of Plaque Formation on Cell Lines of Macrophage Origin—A Sensitive Assay for Antiviral Antibody. *J. gen. Virol.*, **57**, 119.

Penney, J. B. & Wolinsky, J. S. (1979) Neuronal and Oligodendroglial Infection by the WW Strain of Theiler's Virus. *Lab. Invest.*, **40**, 324.

Rabinowitz, S. C. & Lipton, H. L. (1976) Cellular Immunity in Chronic Theiler's Virus Central Nervous System Infection. *J. Immunol.*, **117**, 357.

Roos, R. P., Firestone, S., Wollmann, R., Variakojis, D. & Arnason, B. G. W. (1982) The Effect of Short-Term and Chronic Immuno-Suppression on Theiler's Virus Demyelination. *J. Neuroimmunol.*, **2**, 223.

Schlesinger, J. J. & Brandriss, M. W. (1981) Growth of 17D Yellow Fever Virus in a Macrophage-Like Cell Line, U937: Role of Fc and Viral Receptors in Antibody-Mediated Infection. *J. Immunol.*, **127**, 659.

Traugott, U., Snyder, S. & Raine, C. S. (1979) Oligodendrocyte Staining by Multiple Sclerosis Serum is Nonspecific. *Ann. Neurol.*, **6**. 13.

Yam, L. T., Li, C. Y. & Crosby, W. H. (1971) Cytochemical Identification of Monocytes and Granulocytes. *Am. J. clin. Pathol.*, **55**, 283.