Demyelination in Mice Resulting from Infection with a Mutant of Semliki Forest Virus*

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Summary. Twelve of 34 weanling mice (35%) developed lesions in the brain and spinal cord following i.p. infection with 10^2 p.f.u. of a mutant of Semliki Forest virus (SFV). Six of 12 mice examined 13 days post infection (p.i.) showed meningo-encephalomyelitis with focal spongiform lesions in the grey and white matter. The spongiform lesions were characterised by necrosis of putative oligodendrocytes, myelinic vacuolation and mononuclear cell infiltration. Only one of six mice examined 21 days p.i. and one of six mice examined 28 days p.i. showed lesions which comprised reactive and dystrophic changes in the white matter. Spongiform lesions and pycnotic nuclei were not seen at these times. Viral nucleocapsids were seen in the early stages of the disease in putative necrotic oligodendrocytes. Mature virus particles were not seen. This was in contrast to mice infected with virulent wild-type SFV when lesions were more severe and were accompanied by large numbers of immature and mature virus particles. It is suggested that the demyelination in mice infected with mutant SFV results primarily from selective destruction of oligodendrocytes by the mutant virus.

Key words: Semliki Forest virus — Demyelinating diseases — Spongy degeneration — Oligodendrocytes

Semliki Forest virus (SFV), an alphavirus of the Togaviridae, was first isolated from a pool of mosquitoes in Uganda in 1942 (Smithburn and Haddow

1944). The relative virulence of different strains of this virus has been characterised (Bradish et al. 1971, 1972; Bradish and Allner 1972). The virulent wild-type (wt) strain causes a fatal encephalitis in young mammals. The avirulent A 774 strain results in lesions similar to those caused by the wt strain but, in contrast, this infection is usually subclinical and the lesions subsequently regress (Chew-Lim et al. 1977a).

Demyelination is a feature of infection with the avirulent strain of SFV virus. The pathogenetic mechanism of myelin destruction is poorly understood and some of the evidence which has been presented is contradictory. Chew-Lim et al. (1977b) showed that the efficient production of antibody is protective against demyelination in single dose infections with A 774-SFV. Mice receiving 500 rad total body irradiation prior to infection showed multiple foci of demyelination, a delayed antibody response and yielded the highest titre of virus. Similar results were obtained when the same strain of virus was used in athymic nude mice (Chew-Lim 1979). These authors concluded that demyelination in A 774-SFV infection probably results from direct viral activity rather than from an immunological reaction. Ultrastructural studies of mice infected with A 774-SFV showed no changes in oligodendrocytes (Chew-Lim et al. 1978; Suckling et al. 1978). It seemed unlikely, therefore, that direct viral damage to the oligodendrocyte was a cause of the demyelination plaque. In contrast to the foregoing Jagelman et al. (1978) found no evidence of demyelination in athymic nude mice infected with A 774-SFV while focal areas of demyelination were seen in up to 26% of similarly infected heterozygous and conventional Swiss A₂G mice. It was suggested from this study that an immunological response related to the presence of thymus-derived lymphocytes was involved in the development of demyelination in immunocompetent animals. Suckling et al. (1978) found that the incidence of myelin loss varied from 8–21% when some strains

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of mice, other than Swiss A₂G, were infected with A 774-SFV. These findings indicated that the genetic constitution of the mice also influenced the pathogenesis of the disease.

We have investigated the properties of SFV which determine virulence by subjecting a single strain of SFV of known and high virulence to a chemical mutagen and isolating mutants showing altered virulence (Barrett et al. 1980). Of three such isolated mutants only one (M 136) was able to enter and multiply in the brain following i.p. infection with 10^2 p.f.u. This dose was lethal for the wt strain. Most mice infected with the mutant M 136 survived although a proportion showed demyelination at 13 days after infection when no infectious virus was present (Barrett et al. 1980). In this paper we characterise these lesions further and suggest that the demyelination is due to the selective destruction of oligodendrocytes by the mutant virus.

Material and Methods

Virus

Properties of the virulent L 10 strain of SFV and the isolation of the mutant M 136 have been described (Barrett et al. 1980).

Experimental Animals

Weanling (30–40 days old) inbred mice of the Balb/c strain were injected i.p. with 0.5 ml of BHK growth medium containing 10^2 p.f.u. of wild-type (wt) or mutant virus. Duplicate mice infected with wt-SFV were killed 3, 4, 5, and 6 days after infection (p.i.). Thirty-four mice infected with the mutant M 136 were killed: after 3 days (five mice), 5 days (five mice), 13 days (12 mice), 21 days (six mice), and 28 days (six mice) (Table 1). As a control, six mice injected with growth medium alone were killed: after 3 days (two mice), 5 days (two mice), and 13 days (two mice).

Histology and Electron Microscopy

Mice were anaesthetised with ether and perfused via the left ventricle for 15 min with 3% glutaraldehyde buffered to pH 7.2 with phosphate. The animals were left overnight in polythene bags containing fixative at 4°C before the brains and spinal cords were removed. Coronal sections of brain were cut: one from the frontal cortex anterior to the optic chiasma; one from the mid-brain at the level of the anterior colliculi; and one from the medulla at the level of the corpus trapezoidum. Sections were collected from the mid-vermis of the cerebellum and from the cervical and thoracic spinal cords. Tissues were postfixed in phosphate-buffered osmium tetroxide for 2 h, dehydrated, and embedded in Araldite. Thick sections for light microscopy were stained with toluidine blue. Ultra-thin sections from selected areas were collected on uncoated grids, stained with uranyl acetate and lead citrate and examined at 60 kV with an electron microscope.

The remaining pieces of brain and spinal cord were embedded in paraffin wax, sectioned, and stained with haematoxylin-eosin (HE).

Results

Mice infected with wt-SFV became moribund and died quickly between 4 and 6 days p.i. About 30% of mice

Table 1. The proportion of weanling mice with lesions in the CNS following infection with M 136-SFV

Days after infection	No. mice examined	No. mice with lesions
3	5	1
5	5	3
13	12	6
21	6	1
28	6	1

infected with the mutant M 136 showed ruffling of the fur and transient posterior paresis between 8 and 15 days p.i.

Light Microscopy

Wt-SFV. Lesions in the CNS of these mice resembled previous descriptions of virulent SFV infection in mice (Seamer et al. 1967; Mackenzie et al. 1978). The earliest lesions were seen 3 days p.i. when occasional polymorphonuclear leucocytes were present in the leptomeninges, perivascular spaces, and neuropil of the brain and spinal cord. By day 5 p.i. polymorphonuclear leucocytes and mononuclear cells were prominent in these locations. Mice in the terminal stages of the disease showed neuronal degeneration, neuronophagia by polymorphonuclear leucocytes, pyknosis of glial nuclei, spongiform degeneration of grey matter, and Wallerian degeneration in the spinal cord. Foci of necrosis were seen in the grey matter on day 6 p.i. Severely affected areas included the frontal cerebral cortex, thalamus, hippocampus, pons, nuclei of the medulla, and ventral horns of grey matter in the spinal cord. No lesions were seen in the brains and spinal cords of control mice.

M 136-SFV. The results of this study are summarised in Table 1. On day 3 p.i. polymorphonuclear leucocytes and mononuclear cells were observed in the leptomeninges and perivascular spaces in the brain and spinal cord of one of five mice examined. Numbers of pyknotic and karyolytic nuclei were present around neurones and in the white matter (Fig. 1). Three of five mice examined at 5 days p.i. showed inflammatory cells in the leptomeninges and perivascular spaces. Pyknotic nuclei and glial cell aggregates were randomly distributed in the neuropil. Small vacuoles were present in the white matter in some of these areas. Neuronal degeneration and neuronophagia, as in the wt infection, were seen in one of the three mice with lesions at this time. By day 13 p.i., focal areas of spongiform degeneration were randomly distributed at all levels of the brains and spinal cords in six of 12 mice examined. The spongiform lesions varied in size and form and were

Fig. 1. Junction of grey and white matter in the ventral horn of the spinal cord at 5 days p.i. Pycnotic nuclei around a neurone and between myelinated axons. Toluidine blue. $\times 420$

Fig. 2. a Demyelinating plaque in the lateral funiculus of the spinal cord at 13 days p.i. Toluidine blue. $\times 105$. **b** Higher magnification of **a**. Note the vacuolated myelin sheaths, the pycnotic nucleus closely associated with myelinated axons (*large arrow*), and the macrophage containing lipid vacuoles (*small arrow*). Toluidine blue. $\times 1,050$

Fig. 3. Vacuolated myelin sheaths in the medial lemniscus at 13 days p.i. Toluidine blue. $\times 1,050$

Fig. 4. Cerebellum at 13 days p.i. Note the perivascular mononuclear cell infiltrate and the pycnotic nuclei in the white matter. Toluidine blue. $\times 420$

Fig. 5. Ventral funiculus of the spinal cord at 13 days p.i. Note the pycnotic nuclei (*arrows*) and the dystrophic fibres. Toluidine blue. $\times 420$

Fig. 6. A putative necrotic oligodendrocyte in the spinal cord at 5 days p.i. Viral nucleocapsids are present in the cytoplasm (arrows). $\times 15,400$

Fig. 7. Paracrystalline arrays of viral nucleocapsids in a putative necrotic oligodendrocyte. $\times 15,785$. *Inset:* Higher magnification of viral nucleocapsids. $\times 34,650$

present in white and grey matter (Figs. 2a, 3). Macrophages and pycnotic nuclei were closely associated with these lesions (Fig. 2b). Mononuclear cell infiltrates were prominent in the leptomeninges and perivascular spaces (Fig. 4). Some cells in the perivascular spaces contained myelin debris. The spinal cords showed dystrophic fibres and compressed myelin sheaths particularly in the ventral and lateral funiculi (Fig. 5). One of six mice examined at 21 days p.i. and one out of six mice examined at 28 days p.i. showed dystrophic fibres in the white matter of the spinal cord. Occasional mononuclear inflammatory cells were present in the leptomeninges and perivascular spaces. No pycnotic nuclei or spongiform lesions were seen at this time.

Electron Microscopy

Wt-SFV. Lesions resembled those described previously in mice infected with virulent SFV (Grimley and Friedman 1970; Pathak and Webb 1974, 1978).

Virus particles were seen on day 5 p.i. as rows in the intercellular spaces and as aggregates in areas of necrosis. Developmental stages of the virus were present in neurones associated with cytoplasmic vesicles. Neurones also showed loss of polyribosomes, widening of the perinuclear cistern, and arrays of intermeshed tubular profiles. Vesicles containing virus particles were seen occasionally in the cytoplasm of macrophages. Macrophages containing myelin debris were prominent. The spongy degeneration seen with the light

Fig. 8. Spinal white matter at 13 days p.i. Putative necrotic oligodendrocyte surrounded by the outermost lamellae of a large myelinated axon. $\times 13,300$

Fig. 9. Spinal white matter at 13 days p.i. Advanced lysis of a putative necrotic oligodendrocyte. Note the apparent continuity with the outermost lamella of the adjacent myelin sheath (arrow). $\times 12,840$

microscope was characterised by swelling of astrocytic processes and vesicular disruption of myelin sheaths. Occasional small necrotic cells with condensation of nuclear chromatin and clumped cytoplasmic organelles occurred as satellites to neurones and in the white matter between myelinated axons.

M 136-SFV. On day 5 p.i. cells with pycnotic nuclei, as seen by light microscopy, were small, dense amorphous cells closely associated with myelinated axons. Some of these cells contained paracrystalline arrays of viral nucleocapsids in the cytoplasm (Figs. 6, 7). The viral nucleocapsids averaged about 25 nm in diameter. Mature virus particles were not seen.

On day 13 p.i. cells with pycnotic nuclei were more numerous. Continuities were seen between some of these necrotic cells and adjacent myelinated axons (Fig. 8). The nuclear chromatin of these cells was condensed and the nuclear membrane was indistinct. Cytoplasmic organelles were scanty but included smooth surfaced vesicles and degenerate mitochondria (Fig. 9). The vacuoles seen by light microscopy were within myelin sheaths and between myelin sheaths and axons. The vacuoles varied greatly in size and shape with some up to 30 μm in diameter. Most vacuoles were empty. Others contained loose whorls of myelin or myelin strands stretching across the vacuoles (Fig. 10). Vacuolation of the outermost lamellae of some myelin sheaths had resulted in compression of the sheaths around degenerate axoplasm (Fig. 11).

Astrocytic processes in areas of spongiform change were often swollen (Fig. 10) and macrophages with myelin debris, 'lyre bodies', and lipid vacuoles were frequent (Fig. 12). Enlarged reactive axons in areas of spongiform change were packed with mitochondria, laminated dense bodies, and vesicles (Fig. 13). Similarly enlarged axons and compressed myelin sheaths were seen in two of the 12 mice examined on days 21 and 28 p.i.

Discussion

The results of the present study have shown that lesions were induced in 35% of Balb/c mice following i.p. infection with the M 136 mutant of Semliki Forest virus.

The lesions which resulted from infection with the M 136 mutant differed from those caused by the virulent wt virus in three important respects. Firstly, virus particles were seen only in the early stages of M 136 infection in contrast with the wt lesions where viruses were always present in large numbers. These findings correlated with the virus isolation studies which showed that the M 136 mutant entered the brain only transiently, being cleared by day 4 p.i., as opposed to the wt virus which persisted in the brain until death (Barrett et al. 1980). Secondly, spongiform lesions developed more slowly and were more focal in the M 136 lesions. Thirdly, neuronal necrosis was widespread in the wt infection as opposed to the M 136

Fig. 10. An area of medulla oblongata at 13 days p.i. Vacuoles in the myelin sheaths and between myelin sheaths and axons. Astrocytic processes (*As*) are swollen. Note the macrophage processes containing myelin debris (*D*) and lipid vacuoles (*V*). $\times 5,865$

infection where necrotic change remained confined to cells which closely resembled oligodendrocytes. The interfascicular and perineuronal distribution of these cells and the continuity with adjacent myelin sheaths constituted additional evidence for the oligodendrocyte nature of these cells. Necrosis of oligodendrocytes and putative oligodendrocytes also has been described in experimental allergic encephalomyelitis (Lampert 1967); JHM murine corona virus encephalomyelitis in mice (Lampert et al. 1973) and rats (Nagashima et al. 1978); and cuprizone intoxication in mice (Blakemore 1972). The occurrence of paracrystalline arrays of virus particles in the necrotic cells 5 days p.i. in the present study suggested that the necrosis resulted from infection with the M 136 mutant.

The necrotic changes in the CNS of mice infected with the M 136 mutant contrasted with the findings in mice infected with the avirulent A 774 strain where cytotoxic effects directly attributable to virus replication have not been found (Suckling et al. 1978). Mechanisms of demyelination in viral infections can be divided into those where the cytotoxic effect of the virus is directly responsible for destruction of the myelinating cell and those where the demyelination is immu-

nologically mediated (Wisniewski 1977). Examples of the cytotoxic type of demyelination include acute murine encephalomyelitis caused by the JHM strain of mouse hepatitis virus (MHV) (Lampert et al. 1973). Theiler's virus, another cause of encephalomyelitis in mice, has been proposed as a model system for the study of immunologically mediated demyelination (Dal Canto and Lipton 1977). Demyelination occurs as a late manifestation of this disease, it is accompanied by an inflammatory cell infiltrate and can be prevented by immunosuppressive treatment. Recent studies with the WW strain of Theiler's virus in newborn mice have shown lysis of oligodendrocytes during acute infection (Penney and Wolinsky 1979). The possibility was considered that oligodendroglial antigens released during this phase could constitute a stimulus for the subsequent autoimmune demyelination. The putative necrotic oligodendrocytes in mice infected with M 136-SFV were accompanied by occasional mononuclear inflammatory cells on day 5 p.i. Inflammatory cells were more numerous on day 13 p.i. when the necrotic cells were also accompanied by myelin breakdown. Stripping of myelin lamellae by invading mononuclear cell processes (Wisniewski 1977) was not seen, however,

Fig. 11. An area of medulla oblongata at 13 days p.i. Intramyelinic vacuolation and a compressed myelin sheath. $\times 9,100$

Fig. 12. Spinal white matter at 13 days p.i. A macrophage (*M*) containing myelin debris and lipid vacuoles. $\times 5,250$

Fig. 13. An area of medulla oblongata at 13 days p.i. A reactive axonal enlargement with dense membranous bodies, mitochondria, and vesicles. $\times 8,750$

and the lesions apparently resolved quickly without any evidence of a secondary wave of demyelination. These findings suggest that the mechanism of demyelination in mice infected with M 136-SFV is primarily that of a cytolytic effect on oligodendrocytes with myelin breakdown secondary to oligodendrocyte destruction. Further studies are required before the significance of any immunopathological mechanism in this infection can be assessed.

Occasional dystrophic fibres and reactive axonal enlargements in the white matter accompanied by mononuclear inflammatory cells in the leptomeninges

and perivascular spaces constituted the only lesions in one of six examined 21 days p.i. and one of six mice examined 28 days p.i. with M 136-SFV. No evidence of pycnotic nuclei or spongiform degeneration was seen at these times and the ten remaining mice appeared normal. Resolution has been shown to follow the demyelinating lesions caused in mice by JHM-MHV (Herndon et al. 1975) and A 774-SFV (Chew-Lim et al. 1977a). Mice surviving to the 3rd week after infection with JHM-MHV showed evidence of remyelination and at the end of 3 months demyelinated areas were difficult to detect. Electron-microscopic autoradio-

graphic studies with ^3H -thymidine showed that this remyelination was associated with newly generated oligodendrocytes (Herndon et al. 1977). Studies are in progress to investigate the resolution of the lesions caused by M 136 and other mutants of SFV.

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