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Citation for published version:

Document Version:
Publisher's PDF, also known as Version of record

Published In:
British journal of experimental pathology

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Semliki Forest virus induced, immune mediated demyelination: the effect of irradiation

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Received for publication 2 June 1986
Accepted for publication 18 September 1986

Summary. Intraperitoneal infection with the avirulent A7(74) strain of the alphavirus Semliki Forest virus (SFV) induces an immune mediated demyelinating encephalomyelitis. The blood and brain virus titres, the serum antibody titres and the histopathological changes in the brains of normal mice and mice immunosuppressed with 5.0 or 8.0 Gy total body irradiation (TBX) were determined. SFV infection of immunosuppressed mice resulted in persistently high blood and brain virus titres, neuronal pycnosis, paralysis and death. No demyelination or central nervous system (CNS) inflammatory response occurred in these immunosuppressed mice despite high and persistent brain virus titres. The CNS inflammatory response and associated demyelination could be restored to infected immunosuppressed mice by adoptive transfer of spleen cells, and these changes were brought forward if the donor spleen cells were from mice previously sensitized to SFV. The results indicate that the immune response following SFV A7(74) infection is both protective and pathogenic, and that the demyelination is immune mediated and does not result from direct viral destruction of oligodendrocytes, or any other direct effect of the virus.

Keywords: demyelination. Semliki Forest virus. immunosuppression. irradiation

Immunosuppression as a means of investigating the relationship between viral infection, immune response and pathology has been used for many years and studied in many experimental viral infections (reviewed by Nathanson & Cole 1971). Immunosuppression by irradiation has been used in the study of several infections including: lymphocytic choriomeningitis virus (Rowe 1954), encephalomyocarditis virus (Murphy & Glasgow 1968), Langat virus (Webb et al. 1968), and Sindbis virus (Park et al. 1980).

Semliki Forest virus (SFV) is an alphavirus of the Togaviridae. Intraperitoneal infection of the mouse with the avirulent A7(74) strain (Bradish et al. 1971) results in: a viraemia (Fleming 1977), replication of virus in several tissues including the brain (Pusztai et al. 1971), a transient disturbance of the blood brain barrier (Parsons & Webb 1982a) and production of antibody within the CNS (Parsons & Webb 1984). Histopathological examination of the brain reveals a demyelinating meningo-encephalo-myelitis (Chew-Lim 1975; Mackenzie et al. 1978; Kelly et al. 1982), with an associated pleocytosis (Parsons & Webb 1982b). The spinal cord (Pathak et al. 1983) and optic nerves (Illavia et al. 1982) are also involved, and there are demyelination associated neurophysiological abnormalities of the optic nerve (Tremain & Ikeda 1983; Pessoa & Ikeda, 1984).

Several investigations have attempted to
determine the cause of the demyelination, and whether this results directly from viral damage or from the inflammatory response initiated by the infection. Chew-Lim et al. (1977), in a complicated experiment, studied the effect of 5.0 Gy total body irradiation on the outcome of A7(74) infection following three inoculations of the virus, and concluded that demyelination was related directly to viral activity and not to the immune response. Contrary to this finding, Jagelman et al. (1978) determined that, despite prolonged brain virus in infected athymic nude mice, demyelination did not occur, indicating that demyelination is immune mediated. That demyelination is immune mediated is supported by the production of demyelination in infected nude mice reconstituted with normal or with SFV sensitized spleen cells (Fazakerley et al. 1983). In consideration of the finding that demyelination in the nude mouse is immune mediated and is not a direct result of infection, it was decided to investigate in more detail the immunosuppressive effect of whole body irradiation on the course of SFV A7(74) infection.

Materials and methods

Mice. Inbred male mice, 4 to 6 weeks old were from a colony of Swiss/A2G mice bred at St Thomas' Hospital Medical School, London.

Virus and virus titres. The avirulent A7(74)/C2 strain of SFV (Bradish et al. 1971) was used. On killing of the mice, blood samples were diluted 1:10 in phosphate buffered saline containing 0.75 bovine serum albumin (BAPS) and half brains for assay were stored at -70°C until use. The virus infectivity of a sample was determined by preparing serial 10-fold dilutions in sterile BAPS and inoculating 0.02 ml of an appropriate range of dilutions intracerebrally (i.c.) into groups of four to six suckling mice, 0–4 days old. The i.c. 50% lethal dose/0.02 ml (ICLD50) was calculated by the method of Reed and Muench (1938). Mice were inoculated intraperitoneally 24 h after irradiation with 104.5 ICLD50 of virus, in 0.1 ml of BAPS.

Antibody titres. An enzyme-linked immunosorbent assay (ELISA) was used to measure anti-SFV IgG levels. The assay was based on that of Voller et al. (1976). Virus was purified by a modification of the method of Bruton and Kennedy (1976), and coated to the bottom of Dynatech PVC microtitre plates (Dynatech Laboratories Ltd, Daux Road, Billinghamurst, Sussex). Serum samples were diluted 1/200 in PBS+5% FCS (fetal calf serum) and run in triplicate. Goat antimouse IgG (Fc IgG1/2a/2b/3) linked to horse radish peroxidase, was used as the conjugate (Nordic Immunological Reagents, Maidenhead, Berks). The substrate was o-phenylenediamine (Sigma Chemicals, Poole, Dorset). The difference in absorbance at 492 nm and 690 nm was measured and expressed relative to that of a known positive—a pooled post inoculation day (PID) 21, anti-SFV serum, which was run in triplicate on each plate. The intraplate coefficient of variation was 8% and the interplate 12%.

Histology. Half brains were placed immediately after removal in 5% formol saline, and processed using standard histological techniques. The brains were sectioned sagitally and 5 µm sections were cut from three different areas of each brain. Two sections from each of these three areas were stained with haematoxylin and eosin and two sections with luxol fast blue. A total of 12 sections was thus examined from each brain. Sections were coded, examined in random order and scored on a scale of + to ++++ according to increasing degree of severity of the lesions, for meningitis (MEN), perivascular cuffing (PVC), microcystic change (MCC) and demyelination (DEM).

Irradiation. Mice were placed in a perspex box specially designed to ensure an even dose of irradiation to all the mice within. The mice each received 5.0 or 8.0 Gy total body gamma irradiation (TBX) from a 60Co Mobal-
tron radiotherapy machine. The machine had two opposed sources, with a 90 cm source-to-axis distance, a beam size of 24 × 24 cm, and a dose rate of approximately 0.65 Gy/min.

All mice given 8.0 Gy were given 2 × 10⁷ bone marrow cells intravenously within 4 h of irradiation. The bone marrow cells were prepared from the femurs of syngeneic mice.

Irradiated mice were kept in a filtered isolator and provided with sterile feed and water. The water contained antibiotics, 10 mg/ml neomycin (mycifradin sulphate) and 1 mg/ml polymyxin (aerosporin), and was acidified to pH 3.5 by the addition of a few drops of 2M HCl.

Adoptive transfer of cells. Spleen cell suspensions were prepared from syngeneic mice. These cells had a viability > 90% and each mouse received 5 × 10⁷ nucleated splenocytes in 0.5 ml of RPMI-1640 media (Flow Laboratories) intraperitoneally 24 h post-infection. Mice were hyperimmunized to the virus by giving three doses of 10⁴.5 ICLD₅₀ of SFV at 0, 14 and 21 days. Spleen cells were removed from hyperimmune mice 7 days after the last inoculation of virus.

Determination of mean survival time. The mean survival time (t) was calculated according to the formula of Semenov et al. (1975).

\[ \frac{1}{t} = \frac{1}{t_1} + \frac{1}{t_n} \]

where 't₁' is the time of death of the first mouse and 'tₙ' that of the last mouse. N is the total number of animals. Animals surviving 21 days were deemed to have survived and were not included in the calculation.

Results

The normal infection of S/A2G mice

Mice were inoculated with virus and two mice sampled on each post-inoculation day (PID), 1 to 12. The blood and brain virus titres and the serum antibody levels are recorded in Fig. 1.

Histological examination of the brains of these mice demonstrated by PID 4, a mononuclear cell meningitis and mononuclear cell perivascular cuffing of some of the vessels. From PID 4 onwards the extent of the cuffing around the vessels, and the number of vessels involved increased. Mononuclear cells could be seen leaving some of the vessels or cuffs and invading the surrounding tissues, this was most obvious from PID 7 onwards and

Fig. 1. Blood and brain virus titres and serum IgG anti-viral antibody titres following intraperitoneal inoculation of SFV.
Fig. 2. Perivascular cuffing (P), microcystic changes (C), invading mononuclear cells (M) and demyelination in a cerebellar tract. PID 12 after SFV. Luxol fast blue, cresyl violet, × 360.

Fig. 3. Lesions of demyelination in the cerebellum. PID 14 after SFV. Luxol fast blue, cresyl violet, × 96.
was associated with a microcystic degeneration of the invaded white matter (Fig. 2). Demyelination was occasionally seen by PID 9, but was most apparent between PID 14 and 21 (Fig. 3). The lesions of demyelination were always associated with invading mononuclear cells (Fig. 4). No pycnosis or loss of granule cells (Fig. 5) or oligodendrocytes (Fig. 6) was apparent in the non inflammatory areas of the brains of these mice. In the areas of microcystic change and inflammation it was impossible by light microscopy to determine the integrity of these cells, and it is possible that loss of neurones and oligodendrocytes occurs in such areas (Figs 2 & 4).

The effect of 5.0 Gy irradiation

Forty-five mice were given 5.0 Gy TBX, 24 h before SFV A7(74) infection. Five mice were sampled for determination of the blood, brain and antibody titres, as shown in Fig. 7. Table 1 summarizes the histopathological changes.

Meningitis and perivascular cuffing, apparent in all the control mice by PID 4, were not observed in the infected mice given 5.0 Gy until PID 9, and then only in 2/5 of these mice. The development of the microcystic changes was also delayed, however by PID 18 these were at least as extensive as those seen in the control infection. Few inflammatory cells were associated with the microcystic change. In some areas of the white matter microcysts ran in lines between and parallel to the fibres (Fig. 8). Following 5.0 Gy TBX the onset of demyelination was not delayed in all the mice, but when present was less intense between PID 12 and 18 than in the mice given virus alone; demyelination comparable to that seen in the control mice was not seen in the 5.0 Gy irradiated mice until PID 21. Slight pycnosis of the granule cells of the cerebellum was apparent from...
Fig. 5. Granule cells of the cerebellum are not damaged during the normal infection. PID 7 after SFV. H & E. × 360.

Fig. 6. Chains of normal healthy oligodendrocytes in the white matter. PID 7 after SFV. H & E. × 360.
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Table 1. Pathological changes in the CNS following 5.0 Gy TBX

<table>
<thead>
<tr>
<th>PID</th>
<th>MEN</th>
<th>PVC</th>
<th>MCC</th>
<th>DEM</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>+(2/5)</td>
<td>+(2/5)</td>
<td>+(1/5)</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+(3/5)</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>+(3/5)</td>
<td>++</td>
<td>+(2/5)</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

Each figure represents the mean of five mice. Figures in parentheses indicate the number of mice in the group that were positive, if not shown, all the mice in the group were positive/negative (as indicated).

MEN, meningitis; PVC, perivascular cuffing; MCC, microcystic change; DEM, demyelination.

The effect of 8.0 Gy irradiation

Twenty-five mice were given 8.0 Gy TBX and syngeneic bone marrow cells. Fifteen mice were infected with virus 24 h later, the remaining 10 mice remained uninfected. Two of these uninfected mice died (one on PID 9, and one on PID 13), two mice were sampled on each of days 5, 7 and 9 post-irradiation, the remaining two mice survived for at least 8 weeks after irradiation. The mice sampled were the most sick looking mice on each day. The infected irradiated mice looked thin, hunched with ruffled fur and had paresis of the limbs by PID 7. By PID 9 some of the mice, and by PID 12 all of the mice, had complete paralysis of the front and hind limbs and were moribund. Three mice died (two on PID 15 and one on PID 20) and two mice were sampled on each of PID 4, 5, 7, 9, 12 and 14; on each day the most sick looking mice were sampled.

Figure 9 shows the blood and brain virus titres. No antiviral IgG antibodies were detectable by ELISA, and no inflammation, microcysts or demyelinations were present in any of the brains; with the exception of a slight meningitis in 1/2 mice on PID 12 and 1/2 mice on PID 14. This can probably be attributed to a partial recovery of the immune system by this time. Pycnosis and loss of granule cells (Fig. 10, cf. Fig. 5), was apparent in the brains of all the infected mice examined on PID 7, 9, 12 and 14, and was

PID 3 onwards, but was most obvious on days, 5, 7 and 9.

Fig. 7. Blood and brain virus titres and serum IgG anti-viral antibody titres, in mice given 5.0 Gy TBX. 24 h before SFV infection.
much more extensive than that seen following 5.0 Gy TBX. Neuronal loss was also apparent in other areas, such as the hippocampus. Loss of neurones was rarely seen in the mice given irradiation alone, and this was minimal compared to that seen in the infected irradiated mice.

Adoptive transfer of cells to irradiated mice
Mice were infected with virus 24 h after 8.0 Gy TBX and bone marrow transfer, and reconstituted with spleen cells from syngeneic donors 24 h after infection. The mice received spleen cells from either, normal uninfected mice (group A), or from mice hyperimmunized to the virus (group B). Two mice were sampled from each group on each of PID 7, 9 and 14. The results are shown in Table 2.

The SFV sensitized cells produced high levels of antiviral IgG (Table 2) which probably prevented the brain virus titres from rising to lethally high levels. The brain virus was cleared in these mice by PID 9, earlier even than in normal infection (Fig. 1). Following transfer of normal spleen cells 24 h post-infection, by PID 14 no antiviral IgG was detectable in the sera the viraemia persisted until PID 9 and the brain virus titres were only cleared in 1/2 mice on PID 14. It is likely that the transferred spleen cells were not sufficiently active or numerous to prevent brain virus titres rising to lethally high titres.

Average day of death
Table 3 shows the average day of death of 't' for groups of mice given various treatments. In all cases irradiation was given 24 h pre-infection and spleen cells 24 h pre-infection and spleen cells 24 h post-infection. None of the mice in these groups were sampled for other studies.

SFV infection of the irradiated mice was 100% lethal with an average day of death of 8.0. Transfer of normal spleen cells 24 h post infection did not alter this outcome, though transfer of virally sensitized spleen cells

Fig. 8. Microcysts in the white matter. PID 9 after SFV. H & E. × 360.
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Fig. 9. Blood and brain virus titres in mice given 8.0 Gy TBX, 24 h before SFV infection. No serum anti-viral IgG was detectable.

reduced the mortality to 19% and slightly delayed the average day of death.

Discussion

The A7(74) strain of SFV has been shown to replicate predominantly in neurones (Zlotnik & Harris 1970), but also in oligodendrocytes (Pathak et al. 1983). Damage to neurones has been observed following infection with the virulent L10 strain of SFV (Pathak et al. 1976; Barrett et al. 1980), and the avirulent A8 strain (Zlotnik et al. 1972), but not following infection with the A7 or A7(74) strains. The A7 strain seems to have reduced cytopathogenicity for neurones (Atkins 1983). The integrity of the oligodendrocytes following A7(74) infection of normal control mice has not been previously studied, though probable loss of these cells has been shown in the brains of mice infected with the mutant M9 and M136 strains (Sheahan et al. 1981; 1983). In the present study, outside the areas of inflammation, no loss or pycnosis of oligodendrocytes was seen in mice infected with the A7(74) strain of SFV, and none was apparent in an electron microscopic study of these brains (personal communication Dr S. Pathak).

Blood and brain virus titres in the control infected mice were similar to those found by other workers using the A7(74) strain of SFV (Oaten et al. 1976; 1980; Suckling et al. 1976; 1977). Histopathological study of the brains of these mice confirmed the findings of the previous studies by Chew-Lim (1975), Chew-Lim et al. (1977), Suckling et al. (1978), Mackenzie et al. (1978) and Kelly et al. (1982).

Compared to the infection of the normal non-immunosuppressed Swiss/A2G mice (Fig. 1), the blood and brain virus titres (Fig. 7) of the infected mice given 5.0 Gy TBX were prolonged, whilst the production of viral specific IgG and the onset of the CNS inflammatory response (Table 1) were delayed. No deaths resulted in these mice. These findings are in agreement with the irradiation studies of Smillie et al. (1973) and Chew-Lim et al. (1977). Chew-Lim et al. (1977) also examined the brain pathology and observed an increase in the incidence of demyelination and occasional foci of degenerate neurones in the cerebellum. Though not directly comparable to those of Chew-Lim et al. (1977), the experiments reported here demonstrate that a single dose of 5.0 Gy irradiation delays and decreases both the inflammation and the demyelination resulting from a single inoculation of virus.

Immunosuppression with 8.0 Gy TBX
Fig. 10. Pyknosis of granule cells in the cerebellum. PID 7 after SFV, mouse received 8.0 Gy TBX, 24 h before infection. H & E, × 360 (cf. Fig. 5).

Table 2. Adoptive transfer of normal and hyperimmune spleen cells to mice immunosuppressed with 8.0 Gy TBX

<table>
<thead>
<tr>
<th>Mouse</th>
<th>PID</th>
<th>Blood virus</th>
<th>Brain virus</th>
<th>Serum IgG</th>
<th>MEN</th>
<th>PVC</th>
<th>MCC</th>
<th>DEM</th>
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<td>7</td>
<td>4.0</td>
<td>9.0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A2</td>
<td>7</td>
<td>3.8</td>
<td>8.2</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>A3</td>
<td>9</td>
<td>4.2</td>
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<td>0.703</td>
<td>+</td>
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Group A: 8.0 Gy TBX, A7(74) and normal spleen cells.
Group B: 8.0 Gy TBX, A7(74) and hyperimmune spleen cells.
MEN, meningitis; PVC, perivascular cuffing; MCC, microcystic change; DEM, demyelination.
Table 3. Average day of death

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Deaths</th>
<th>t</th>
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<tr>
<td>8.0 Gy TBX(bmc)</td>
<td>8/25</td>
<td>10.4  (8-13)*</td>
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<tr>
<td>8.0 Gy TBX(bmc)+SFV</td>
<td>25/25</td>
<td>8.0 (5-19)</td>
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<tr>
<td>8.0 Gy TBX(bmc)+SFV + normal spleen cells</td>
<td>16/16</td>
<td>7.0  (5-14)</td>
</tr>
<tr>
<td>8.0 Gy TBX(bmc)+SFV + hyperimmune spleen cells</td>
<td>3/16</td>
<td>10.2 (9-12)*</td>
</tr>
</tbody>
</table>

* t. Average day of death (days post-inoculation).
  bmc, Bone marrow cells (2 x 10^7/mouse).

Figures in parentheses indicate the days between which the animals died.

Before infection with the avirulent A7(74) strain of SFV resulted in a rapid rise of the brain virus titres to a high level (10 logs by PID 4, Fig. 9). This is to be compared with a normal maximum of 8 logs by PID 5 (Fig. 1). The rapid rise in brain virus titres following 8.0 Gy TBX is similar to that seen following infection with the virulent L10 or V13 strains of SFV. (Pusztai et al. 1971; Oaten et al. 1980). This finding suggests that one difference determining the rate of replication of these two strains of SFV may be a difference in their interaction with the hosts immune system. For a review of this subject see Atkins et al. (1985).

The effects of irradiation on immune cells have been reviewed by Anderson and Warner (1976) and Doria et al. (1982). Radiation results in a rapid interphase death of lymphocytes, 8.0 Gy also destroys the bone marrow cells but at a dose of 5.0 Gy these cells are spared, and the population of circulating T and B lymphocytes is soon replenished from their precursors in the bone marrow. The latter accounts for the temporary delay in both antibody production and the onset of the inflammatory response, following 5.0 Gy TBX. As expected, the immunosuppressive effect of 8.0 Gy TBX was longer lasting and more dramatic on both blood and brain virus titres, which remained high over the 2 weeks studied. No anti-viral IgG was detectable. Some reduction of the blood virus titres did occur from PID 7 onwards, but even 14 days after infection there were over 2 logs of virus in the blood (Fig. 9). In the normal infection the viraemia was cleared by PID 4 (Fig. 1).

Examination of the brains of mice given 8.0 Gy irradiation alone (uninfected) demonstrated no, or only very occasional loss of, or damage to neurones. Some pycnosis of neurones was apparent in the brains of the mice given 5.0 Gy and this was more apparent in those given 8.0 Gy TBX before infection. CNS cells are generally highly radioresistant, except for some occasional long-term effects, no radiation damage is apparent in the brains of rodents given less than 15 Gy irradiation (Hubbard 1980), and loss of oligodendrocytes is seen only several weeks after irradiation in rats given 40 Gy (Hubbard & Hopewell 1979). The loss and pycnosis of neurones in the irradiated and infected mice is thus most likely to result, following immunosuppression, from the greater spread of virus within the brains of these mice to involve more cells, or even susceptible sub-populations of cells. It is likely that the neuronal destruction following high virus titres is first apparent as paralysis and is then responsible for the death of the infected irradiated (8.0 Gy) mice. Paresis was apparent in the 8.0 Gy treated infected mice by PID 7, and some of the mice were paralysed by PID 9.

The absence of demyelination in the brains of the 8.0 Gy immunosuppressed mice, despite the higher and prolonged brain virus titres, clearly demonstrates that the demyelination does not result from direct viral damage but is dependent upon the immune response. This is confirmed by the results of the reconstitution experiments. The results are similar to those obtained by adoptive transfer of cells to athymic nude mice infected with the A7(74) strain of SFV (Fazakerley et al. 1983).

The experimental findings presented in this paper indicate that following immunosuppression with 8.0 Gy TBX, the A7(74) strain of SFV replicates to high titres within
the brain. These high titres result in occasional loss of neurones and foci of granule cell pycnosis, such mice become paralysed and die. No such changes and no deaths occur in normal immunocompetent mice infected with this virus, demonstrating the protective aspect of the immune response. Despite the very high and persistent virus titres in the brains of the irradiated (8.0 Gy) immunosuppressed mice, no demyelination occurs. In contrast infection of normal immunocompetent mice with this virus results in a demyelinating encephalomyelitis; this aspect of the immune response is thus pathogenic. It is likely that anti-viral antibodies are protective, preventing high, damaging and lethal brain virus titres, and that T lymphocytes are pathogenic producing the demyelination. T lymphocytes in close contact with macrophages, astrocytes and demyelinating axons have been described in the spinal cord following SPV A7(74) infection (Pathak et al. 1983).

Acknowledgements

We thank the Multiple Sclerosis Society of Great Britain and Northern Ireland, and the Charitable Funds of St Thomas' Hospital for the financial support of this work.

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