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The Relationship between Axonal Transport of Protein and Demyelination in the Optic Nerves of Mice Infected with Semliki Forest Virus

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(Accepted 8 April 1986)

Key words: Optic nerve — Axonal transport — Demyelination — Semliki Forest Virus — Virus infection

Fast and slow axonal transport of protein have been studied in the optic nerves of mice infected with Semliki Forest Virus (SFV) that causes patchy demyelination throughout the CNS. Intravitreal injections of [³H]proline were given at regular intervals after virus inoculation, the labelled protein in the superior colliculi was then measured after survival periods of 18 h or 10 days, for fast and slow axonal transport studies, respectively. Fast transport studies showed an enhanced amount of protein arriving at the optic nerve terminals (superior colliculus) of the SFV-infected mice prior to the onset of demyelination. In contrast, the slow transport studies showed an enhanced amount of protein at the superior colliculus of the SFV-infected mice during the demyelination period. There was no concomitant increase in labelled protein in the retina at any time after the SFV infection. It is proposed that alteration in the transport of the protein constituents other than major myelin specific components may cause disruption of myelin maintenance in SFV infection.

INTRODUCTION

The influence of the nerve cell body upon the maintenance of the axon was recognized well over a hundred years ago³² and subsequently, the degeneration of both axon and myelin following an axonal lesion led to the suggestion that the axon, and hence the cell body, was influential in maintaining the myelin sheath²³. Nowadays, much is known about the transport of material along the axon, in both anterograde and retrograde directions²⁶ and several interactive pathways between axon, glia and myelin sheath have been revealed²⁷.

A variety of transport abnormalities have been reported in a number of animal models of demyelination, including changes in the rates of transport^{3,24} and in the amount of material transported¹⁶. Demyelination of the optic nerve induced by an RNA virus, Semliki Forest Virus (SFV)^{14,30} was also accompanied by abnormally increased *amounts* of protein transported by both fast and slow axonal transport systems, although the maximum rate of transport was not affected²¹. The maximum difference between

control and demyelinated nerves occurred 18 h and 10 days after intravitreal injection of tritiated proline. These changes were noted in SFV-infected mice taken at the peak time of demyelination, that is 21 days after receiving the first of two intraperitoneal inoculations of the virus (the second inoculation being given 14 days after the first (*ibid.*))²¹.

The following two questions arose from the above observations. Firstly, whether these changes in axonal transport are a consequence of the demyelinating process or do they occur prior to demyelination? Secondly, is the increased protein transported by the optic nerve caused by enhanced uptake and incorporation at the retina? The present study attempted to answer these two questions. A preliminary account of some of the findings has been published¹.

MATERIALS AND METHODS

Animals

Four hundred and eighty male Swiss/A₂G mice of St. Thomas' Hospital Medical School strain were used. At age 4 weeks, 240 control animals received

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0.1 ml i.p. inoculations of bovine albumin phosphate saline (BAPS) and 240 experimental animals received 0.1 ml i.p. inoculations of 10^{-4} SFV A7(74) diluted in BAPS. A further 40 mice (20 controls, 20 SFV-infected) received an additional inoculation 14 days after the first and this 'two-inoculation' group will be considered separately throughout the paper. Previous to each virus inoculation, the viability of the virus was verified by intracerebral inoculations of approximately 0.02 ml 10^{-4} SFV in BAPS into 2–3-day-old suckling mice. Only virus that caused the death of these mice within 2–3 days was used.

Studies on the time course of changes in fast axonal transport along the optic nerve

Every 2 days, from postinfection day (PID) 2 to 22, after the single i.p. inoculations of BAPS or SFV, groups of 20 4-week-old mice (10 control and 10 SFV-infected) were taken for intravitreal injections, i.e. 11 groups of 20 mice in total. Additionally, 10 control and 10 SFV mice that had received two i.p. inoculations (on day 0 and on PID 14) were also taken for intravitreal injections on PID 22, i.e. 22 days after their first inoculation but 8 days after the second inoculation.

For the intravitreal injections, the mice were pretreated with a neuroleptanalgesic, Hypnorm (Janssen Pharmaceuticals, 1.02 mg/kg i.p.) and anaesthetized with ether. Intravitreal injections of 1–2 μ l of L-[3,4(n)- 3 H]proline (Amersham International) in one eye, at a concentration of 1 mCi/ml and specific activity of 40–60 Ci/mmol, were made under an operating microscope using a 10 μ l Hamilton syringe. 33-Gauge Hamilton needles were resharpened to an external tip diameter of 60–80 μ m and were inserted just behind the limbus margin of the eyeball. Injections were given slowly over 2 min to prevent a sudden increase in intraocular pressure and a drop of light silicone oil (Dow Corning 200/100 cs) placed on the cornea to help seal the injection site and assist wound repair⁴. All injections were given between 14.00 and 20.00 h to minimize the effects of diurnal changes reported previously for axonal transport along rat optic nerves²⁵. For the fast transport study the mice were allowed to survive for 18 h following the intravitreal injections, they were then killed and the injected eye and the brain were rapidly removed and fixed in 10% TCA (trichloroacetic acid, BDH Chemicals) for 48 h,

after which little unincorporated radioactive label remains in the tissue¹². Tissues were then washed in distilled water for a further 24 h to remove all the TCA and then prepared for liquid scintillation counting (see later).

Studies on the time course of changes in slow axonal transport along the optic nerve

Similarly, every 2 days, from PID 2 to 22, after the single i.p. inoculation of BAPS or SFV, groups of 20 mice (10 controls and 10 SFV mice) were taken for intravitreal injections, i.e. 11 groups of 20 mice. Additionally, 10 control and 10 SFV mice that had received two i.p. inoculations (on day 0 and on PID 14) were also taken for intravitreal injection on PID 22 after the first inoculation (8 days after the second). The procedure was the same as for the fast transport study except that the mice were allowed to survive for 10 days after the intravitreal injections, before the animals were killed and tissues were removed, fixed and washed as previously described, prior to liquid scintillation counting.

The intravitreal injection technique is difficult in a small eye such as a mouse's eye, and some animals had to be rejected for the following reasons: (1) misplaced injection, e.g. into lens, through the retina into the sclera, or a badly damaged retina; (2) heavy bleeding into the eyeball or behind the retina; (3) leakage of isotope from the eye during or immediately after injection; and (4) postinjection infection of the eye. The most common reason for rejection of tissues was the first, which was usually determined on dissection of the fixed eye. The second and third categories were also used quite frequently to reject tissues, but only 3 eyes were rejected because of infection. The numbers of animals used to compile the results from the total number of animals inoculated are shown in Table I.

TABLE I

Numbers of the total inoculated mice used to compile the results

	One inoculation		Two inoculations	
	CON	SFV	CON	SFV
Fast transport	87/110	84/110	9/10	8/10
Slow transport	86/110	73/110	8/10	6/10

Retinal levels of incorporated protein

The injected eyes of all the animals used in the fast and slow axonal transport experiments were also excised when the mice were killed. The cornea was pierced with a needle to allow passage of fixative and the eyeballs immersed in 10% TCA for 48 h. After this time the retinae were dissected out and placed in distilled water for 24 h to wash away excess TCA. The retinae were then prepared for liquid scintillation counting.

Liquid scintillation counting

After washing, the superior colliculi were dissected out from the brain and they and the retinae were air-dried and weighed on a Mettler HK60 balance to the nearest 0.01 mg. The tissues were then rehydrated, solubilized in Soluene (Packard) and Dimilume-30 scintillation cocktail (Packard) was added to each sample. The amount of radioactivity was assessed by counting the samples in a 1215 Rakkbeta (LKB-Wallac) scintillation counter. Corrections were applied for the volume of [³H]proline injected and for quenching using the external standard channels-ratio method⁹, the counting efficiency for the samples being of the order of 28–34%. The amount of radioactivity in the retinae was expressed as disintegrations per minute per mg of dried tissue (dpm/mg). For the axonal transport studies, since approximately 97% of optic nerve fibres cross in albino mice⁷, the contralateral superior colliculus was considered as the target tissue and the ipsilateral superior colliculus as the control tissue for diffused or blood-borne label. All the axonal transport results are thus expressed in dpm/mg, as the means ± S.E.M. of the contralateral counts minus the ipsilateral counts.

RESULTS

To assist the interpretation of the axonal transport results we have listed the known consequences following an i.p. inoculation of SFV, in Table II, and we will describe our findings on fast and slow axonal transport separately.

Fast axonal transport studies (18 h after intravitreal injection of [³H]proline)

Changes in fast axonal transport at different times after SFV inoculation are shown in Fig. 1. In the con-

TABLE II

Sequelae of an i.p. inoculation of Semliki Forest Virus in 3–4-week-old Swiss/A₂G mice

Days	
1–3	Interferon, neutralizing antibody produced ¹⁷
4–6	↑ White cell count ¹⁸
5–6	↓ Motor activity ²⁸
5–8	↓ Food and water intake, weight loss ²
6–7	↑ CSF albumin levels ¹⁹
7–10	Hind limb paralysis ² , encephalitis ¹⁹
7–14	Perivascular cuffing, microglial reaction ⁵
7–	↑ Lysosomal enzymes ²⁹
8–	Haemagglutinating antibody ¹⁷ , virus-specific IgG ²⁰ , ↑ white cell count ¹⁸
10	Virus no longer detectable ³³
14–21	Onset of demyelination ⁶
14–24	Astrocytic hypertrophy ¹⁷
28	Some remyelination ¹⁵
42	↓ Perivascular cuffing ²⁹ Enzymes at control levels ²⁹

trol mice there is an initial drop in the amount of radioactivity at the optic nerve terminals but this stabilizes by about PID 6. From then until the end of the experimental period (PID 22) the amount of radioactivity in the control mice remained at a fairly constant level of around 3000 dpm/mg. In contrast, in the SFV-infected mice, there was a sharp increase in the amount of radioactivity after PID 6. The level remained significantly enhanced ($P < 0.001$, Fischer *t*-test) from PID 8 (SFV, 5370 ± 400 dpm/mg; CON, 3340 ± 280 dpm/mg) until PID 14 (SFV, 5264 ± 400 dpm/mg; CON, 3372 ± 380 dpm/mg). After this time, from PID 16 onwards, there was no difference

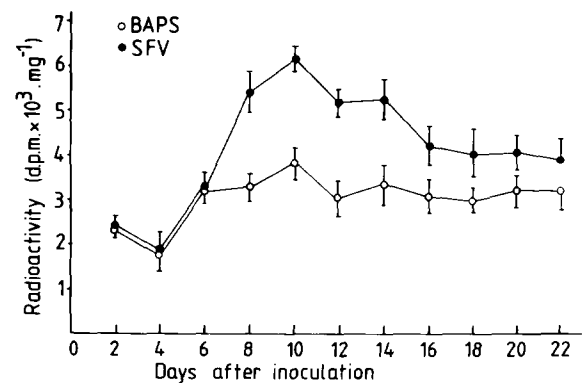


Fig. 1. Fast axonal transport study (18 h after intravitreal injection of [³H]proline) showing the amount of radioactivity in the superior colliculus (contralateral minus ipsilateral counts) against time in days following one i.p. inoculation of either BAPS (open circles) or SFV (closed circles). Each point represents the mean ± S.E.M. of 6–10 samples.

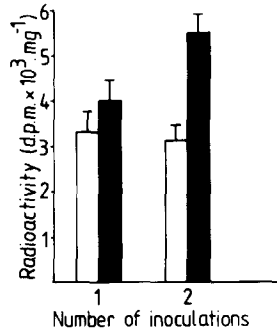


Fig. 2. Fast axonal transport study (18 h after intravitreal injection of [³H]proline) showing the amount of radioactivity in the superior colliculus (contralateral minus ipsilateral counts) of mice 22 days after receiving i.p. inoculations of BAPS (open bars) or SFV (closed bars). The histograms on the left are from animals that had received one inoculation (on day 0) and are redrawn from the PID 22 data in Fig. 1. The histograms on the right are from animals that had received two inoculations (on days 0 and PID 14). Each bar represents the mean \pm S.E.M. of 6–10 samples.

between the amounts of radioactivity in the control and SFV-infected material, following one inoculation of BAPS or SFV.

However, in animals that had received two inoculations there was an increase in the amount of radioactivity in the SFV-infected mice compared with the control mice, as shown in Fig. 2. The histograms on the left are redrawn from Fig. 1 and are the levels of radioactivity in mice on PID 22 that had received one inoculation on day 0. The histograms on the right are the levels of radioactivity in mice taken on PID 22 after two inoculations (on day 0 and PID 14). There was a significant increase in the amount of radioactivity in the superior colliculus of the mice with two SFV inoculations (5500 ± 390 dpm/mg) compared with the controls (3100 ± 400 dpm/mg, $P < 0.001$). There was no difference between the SFV group (4000 ± 425 dpm/mg) and the controls (3275 ± 400 dpm/mg) in the animals with only one inoculation. There was no difference between the control mice that had received one inoculation (3275 ± 400 dpm/mg) or two inoculations of BAPS (3100 ± 400 dpm/mg).

Study of radioactivity in the retina 18 h after intravitreal injection of [³H]proline. The enhancement of radioactivity found at the superior colliculus of the SFV-infected mice might reflect enhanced uptake of the labelled amino acid by the retinal ganglion cells. The levels of incorporated radioactivity remaining in

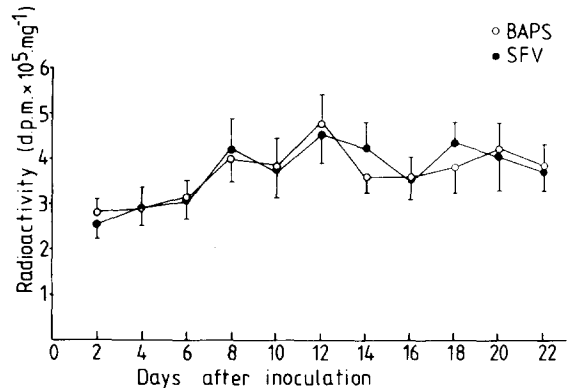


Fig. 3. Levels of radioactivity in the retina (18 h after intravitreal injection of [³H]proline) against time in days following one i.p. inoculation of either BAPS (open circles) or SFV (closed circles). Each point represents the mean \pm S.E.M. of 4–6 samples.

the retina of control and SFV-infected mice are shown in Fig. 3. Whilst there was an initial increase in the amount of radioactivity up to PID 8, there was no difference between control and SFV-infected levels in the retina at any time following inoculation.

Slow axonal transport studies (10 days after intravitreal injection of [³H]proline)

The levels of radioactivity in the superior colliculus due to slow axonal transport were higher than those 18 h after intravitreal injection of [³H]proline. Fig. 4 shows the results of the slow transport study, demonstrating that the amount of radioactivity in the control samples remains at a fairly constant level from PID 2 until PID 22. In contrast, in the infected mice there is a significant increase ($P < 0.025$) in the

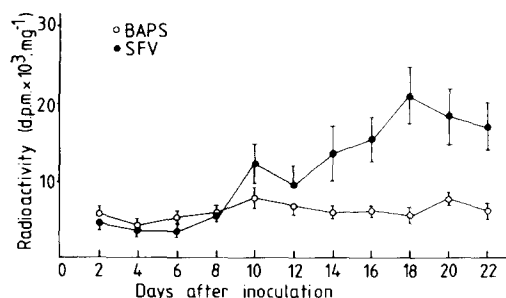


Fig. 4. Slow axonal transport study (10 days after intravitreal injection of [³H]proline) showing the amount of radioactivity in the superior colliculus (contralateral minus ipsilateral counts) against time in days following one i.p. inoculation of either BAPS (open circles) or SFV (closed circles). Each point represents the mean \pm S.E.M. of 6–10 samples.

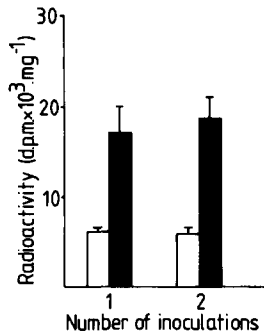


Fig. 5. Slow axonal transport study (10 days after intravitreal injection of [³H]proline) showing the amount of radioactivity in the superior colliculus (contralateral minus ipsilateral counts) of mice 22 days after receiving i.p. inoculations of BAPS (open bars) or SFV (closed bars). The histograms on the left are from animals that had received one inoculation (on day 0) and are redrawn from the PID 22 data in Fig. 4. The histograms on the right are from animals that had received two inoculations (on day 0 and PID 14). Each bar represents the mean \pm S.E.M. of 6–10 samples.

amount of radioactivity by PID 14. This increase reaches a maximum by PID 18 (CON, 5500 ± 860 dpm/mg; SFV, 20900 ± 3580 dpm/mg, $P < 0.001$) and remains significantly higher than control levels at PID 22.

The effects of two inoculations of BAPS or SFV on slow axonal transport are shown in Fig. 5. The histograms on the left are the levels of radioactivity in mice taken on PID 22 that had received one inoculation on day 0. The histograms on the right are the levels of radioactivity in mice taken on PID 22 after two inoculations (on day 0 and PID 14). Both SFV-infected groups have significantly enhanced amounts of radioactively labelled proteins compared with the controls. But there was no difference in the amount of radioactivity in the animals that had received two SFV inoculations (18600 ± 3500 dpm/mg) and those with only one inoculation (16980 ± 3280 dpm/mg). Similarly, there was no difference between the controls that had received one and two inoculations of BAPS (one inoculation of BAPS, 6053 ± 544 dpm/mg; two inoculations of BAPS, 6010 ± 628 dpm/mg).

Study of radioactivity in the retina 10 days after intravitreal inoculation of [³H]proline. The residual levels of incorporated radioactivity in the retinae of control and SFV-infected mice, 10 days after intravitreal injection, are shown in Fig. 6. These levels are

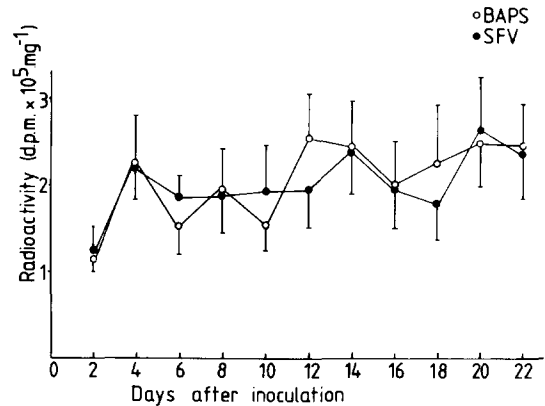


Fig. 6. Levels of radioactivity in the retina (10 days after intravitreal injection of [³H]proline) against time in days following one i.p. inoculation of either BAPS (open circles) or SFV (closed circles). Each point represents the mean \pm S.E.M. of 4–6 samples.

jection as shown in Fig. 3 but there was no difference between the control and infected retinae at any time after inoculation of either BAPS or SFV.

DISCUSSION

In this study we attempted to answer the following two questions: (1) whether the previously reported increase in fast and slow axonal transport of protein along the optic nerve fibres of mice infected with SFV²¹ is a consequence of, or occurs prior to demyelination? and (2) is this increased protein due to enhanced uptake and incorporation of precursors by the retina? The discussion will first therefore be focused upon the answers to these questions.

With regard to the first question, our results suggest that although the change in the slow axonal transport may be a consequence of demyelination, the increase in the fast axonal transport may be a causative factor associated with immunological changes preceding demyelination for the following two reasons.

Firstly, the increase in the amount of protein in the SFV-infected nerve terminals due to slow axonal transport occurred from PID 14 onwards, reaching a peak at PID 18, thus occurring concurrently with the onset of demyelination (see Table II). In contrast, the increased amount of protein in the SFV-infected nerve terminals due to fast axonal transport occurred from PID 8 to 14 (Fig. 1). Since the occurrence of demyelination peaks during PID 14–21 (Table II), this

increase in the fast axonal transport precedes demyelination.

Secondly, the viral specific IgG increase and degree of subsequent demyelination are greater in mice that had received two SFV inoculations than those that received only one inoculation (Illavia and Webb, personal communication). Our observations that the increase in fast axonal transport was much greater in the mice that had two rather than one inoculation of SFV therefore suggests that the increase in the fast axonal transport is related to immunological responses preceding demyelination. On the other hand, since there was no difference in the slow axonal transport between those animals that had one and two inoculations, the change in the slow axonal transport seems to be independent of the immunological responses.

With regard to our question as to whether the increase in axonal transport is due to enhanced uptake and incorporation by precursors by the retina, we found no increase in labelled protein in the retina (measured at 18 h after intravitreal proline injection) at any time after SFV or BAPS inoculation (see Figs. 3 and 5). It is unlikely that the increased axonal transport has been caused by an increased uptake or incorporation by the retinal ganglion cells. The fact that the retinal protein levels remained the same whilst labelled protein accumulated at the optic nerve terminals, leads us to consider the possibilities of decreased turnover of the protein or less utilization of protein along the optic nerve fibres in SFV-infected mice. There is already evidence that membrane properties of optic nerve fibres are greatly disturbed after SFV infection, as the optic nerve fibres produce abnormally enhanced spontaneous firing, as well as long lasting rhythmic oscillatory bursts after stimulation in SFV-infected mice³¹. In addition, SFV is known, from extensive in vitro studies, to affect host cell metabolism and membrane incorporation (e.g. ref. 13). Therefore altered utilization of protein

along the retinal ganglion cell axons would not be surprising. A more detailed study of axonal transport metabolism and energetics following SFV inoculation is awaited. For example, although we already know that the rate of fast and slow axonal transport had not changed at the peak of demyelination²¹, we still need to look at whether the transport rates remained constant throughout after SFV inoculation.

Finally, an important question remains as to the identity of the compounds of the fast axonal transport changes that precede the demyelination induced by SFV. With specific regard to myelin constituents, there is strong evidence for the transfer of lipid material from axon to myelin in the CNS⁸, although studies on protein transfer have yielded more equivocal results. Several authors^{10,11} have isolated both fast and slow axonally transported protein with myelin fractions, and these are identified as high-molecular weight and not the myelin-specific proteins (myelin basic protein, MBP; proteolipid protein, PLP; and DM-20). Since retinal ganglion cells do not synthesize MBP, PLP or DM-20 (ref. 22), it is likely that protein constituents of myelin *other* than the major, myelin-specific components are synthesized and transported along the axons of retinal ganglion cells, and that some alteration of this activity could cause disruption of myelin maintenance in SFV infection. Although this view must remain as conjecture, awaiting further studies, we suggest that neuronal changes are of importance in the study of demyelinating disease.

ACKNOWLEDGEMENTS

This work was supported by grants from the Multiple Sclerosis Society and the Special Trustees of St. Thomas' Hospital to H.I. We are grateful to Dr. Hugh Webb for the gift of SFV, Tim Allen for technical assistance and Gill Blake and Janet Turner for help in preparing the manuscript.

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