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Tumour necrosis factor causes an increase in axonal transport of protein and demyelination in the mouse optic nerve

Huw G. Jenkins and Hisako Ikeda

Vision Research Unit of Sherrington School (UMDS), The Rayne Institute, St. Thomas' Hospital, London (U.K.)

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Summary

An increase in fast axonal transport of protein by the optic nerve was found in mice following a single combined injection of human recombinant tumour necrosis factor alpha (rTNF) and [^{3}H]proline into the vitreous chamber. Demyelination was observed in optic nerve fibres arising from the eyes of mice which received a single rTNF injection. No such changes were detected when heat-inactivated rTNF was injected with the label. The effects of intravitreal injection of rTNF on the pathophysiology of mouse optic nerve resembled those found in mice infected with Semliki Forest virus (SFV), an animal model of multiple sclerosis. We suggest that TNF could mediate at least some of the pathophysiological changes found in SFV-infected mice and may provide a clue concerning the disease mechanism in multiple sclerosis.

Introduction

The maintenance of normal molecular interactions between cells, such as recognition and adhesion, is dependent on proteins and glycoproteins resident in the plasma membrane. In the nervous system, neurons must be able to transfer such proteins from the cell body, over relatively long distances in the axons, to enable their turnover. This is achieved via a fast axonal transport system which continuously shuttles membranous organelles containing proteins and glycoproteins along microtubules (Schnapp and Reese 1986). Changes in components of fast axonal transport could therefore lead to abnormal interaction with myelin, leading ultimately to demyelination.

An association between changes in axonal transport and demyelination has indeed been shown by several studies on animal models of demyelinating disease (Bradley and Jaros 1973; Nagashima et al. 1979; Rao et al. 1981; Pessoa and Ikeda 1984; Ikeda and Tansey 1986). Our laboratory demonstrated a significant increase in the amounts (but not the rates) of proteins and glycoproteins transported to optic nerve endings prior to optic nerve demyelination in mice infected with Semliki Forest virus (SFV) (Tansey and Ikeda 1986; Jenkins and Ikeda 1991; Jenkins et al. 1991), a well-documented model of central nervous system demyelination (Atkins et al. 1985). These events were mediated by the immune system, since T cell-deficient nude mice showed neither increased axonal transport nor demyelination following SFV inoculation (Fazakerly and Webb 1987; Jenkins et al. 1988). Moreover, when nude mice were reconstituted with T cells from immunocompetent *nu* / + littermates, both increased axonal transport and optic nerve demyelination was restored (Jenkins et al. 1988). These results suggest that increased axonal transport and demyelination in SFV-infected mice are both T-cell-dependent, and the axonal transport change is a causative factor for demyelination.

In this paper we asked whether intraocular injection of tumour necrosis factor alpha (TNF), a product of both T-cells (Steffen et al. 1988; Limb et al. 1989) and T cell-activated macrophages, could promote changes in axonal transport and optic nerve demyelination in mice. To our knowledge, the effects of TNF on the visual system have previously only been studied by

Correspondence to: H.G. Jenkins, The Vision Research Unit, The Rayne Institute, St. Thomas' Hospital, London, SE1 7EH, U.K. Tel: (44)(71) 928-9292, ext. 3407; Fax: (44)(71) 922-8079.

Brosnan et al. (1989). These workers found that human recombinant TNF (rTNF) caused a delay in the visually-evoked cortical potential which was associated with inflammatory changes in the rabbit retina. rTNF has been shown to cause myelin damage in organotypic cultures of mouse spinal cord axons (Selmaj and Raine 1988), but the in vivo effect of rTNF on optic nerve myelin does not appear to have been examined. Recently, Simmons and Willenborg (1990) demonstrated that injection of rTNF into rat spinal cord caused an inflammatory response, similar to events observed in experimental autoimmune encephalomyelitis, but its effect on myelin does not appear to have been followed up.

Materials and methods

Animals

Male Swiss/A_2G mice, aged 5–6 weeks, of the St. Thomas' Hospital Medical School strain were used.

Tumour necrosis factor

rTNF at an activity of 10^4 units/ml in culture medium was provided by Dr. K.A. Brown of the Immunology Dept. at this Institute, and stored at −70°C until use. For control experiments, samples of rTNF containing equivalent amounts to each experimental group were heated at 100°C for 5 min.

Axonal transport measurement

Procedures for intravitreal injections and processing of tissues for liquid scintillation counting to study axonal transport in optic nerves have previously been described (Pessoa and Ikeda 1984; Ikeda and Tansey 1986; Tansey and Ikeda 1986). The injections were made into the centre of the vitreous through the superior temporal portion of the sclera at a point approximately 0.7 mm behind the limbus margin, at an angle of 40°–50° with reference to the equator of the eyeball. To prevent a rapid increase in the intraocular pressure and reflux of fluid, the injection was made slowly over a period of 2 min using a fine hydraulically controlled pipette (tip diameter, 60–80 μm) and silicone fluid was applied over the scleral penetration point following the injection. While under deep sodium pentobarbitone anaesthesia (Sagatal, 50 mg/kg; May & Baker Ltd.), experimental mice received 2-μl intravitreal injections containing either: (1) 1 μCi of L-2,3,4,5-[^{3}H]proline stock solution (1 mCi/ml; 122 Ci/mmol; Amersham International plc) together with rTNF into one eye, or (2) rTNF followed by 1 μCi [^{3}H]proline 24 h later. The initial rTNF dose to be tested for its effect on axonal transport was 10 Units. Subsequently, the effects of doses ranging from 0.1 to 10 Units were studied. Control mice received heat-inactivated rTNF together with the radiolabel.

Mice were terminally anaesthetised with ether 18 h after the intraocular injection containing the radiolabel. This corresponds to the time when the accumulation of rapidly transported label reaches a peak level at the contralateral superior colliculus (Pessoa and Ikeda 1984), where 97% of optic nerve fibres terminate in the mouse (Dräger and Olsen 1980).

Eyes and superior colliculi were fixed in 10% (w/v) trichloroacetic acid, washed with water and the retinae dissected. Samples were dried, weighed, and then moistened with 20 μl water before solubilization in 0.3 ml Solusol (National Diagnostics) and addition of 5 ml Fluorosol scintillant (National Diagnostics). A Rakbeta 1215 (LKB-Wallac) counter with quench corrections was used to measure label incorporation. Transported components were calculated using corrections for the blood-borne label, as described previously (Tansey and Ikeda 1986b). Briefly, to make these corrections, the specific radioactivity in the retina from the non-injected eye was subtracted from that in the retina on the injected side. Also, since 97% of optic nerve fibres terminate in the contralateral superior colliculus, blood-borne label in superior colliculi was corrected for by subtracting label found in the colliculus ipsilateral to the injection from that in the contralateral side.

Histology of optic nerves

Eleven days after intravitreal injection, the mice were terminally anaesthetised with ether, optic nerves were rapidly removed post-chiasmally, and processed for histology. Perfusion fixation was not performed because of the technical difficulties inherent in attempts to obtain reproducible perfusion of such small animals, whilst immersion fixation is an acceptable procedure for tissues less than 0.5 mm in diameter such as the mouse optic nerve (Glauert 1975). Histology reagents were obtained from BioRad Microscience Ltd., and aqueous solutions were prepared in ultrapure water. Primary fixation of the optic nerves was carried overnight at 4°C in 2.5% (w/v) glutaraldehyde, 0.1% (w/v) sodium cacodylate, buffered at pH 7.4 with HCl. The nerves were washed in 0.1 M sodium phosphate pH 7.4, for 5 min, and then post-fixed in 1% (w/v) OsO_4 for 2 h. Fixed tissues were dehydrated in an ascending series of alcohol and then acetone before embedding in Araldite.

Approximately 100 semi-thin sections (1.5 μm) were cut from the portion of the optic nerves between 2–4 mm from the optic discs obtained from 3 experimental mice and 3 control mice. These sections were counterstained with toluidine blue and examined by light microscopy. In addition, ultra-thin sections were prepared for investigation using a Hitachi H-7000 transmission electron microscope at 75 kV. All sectioning

and photographic processing of the optic nerves were carried out by the electron microscope unit at UMDS (Guy's Campus).

Results

Effect of intravitreal injection on axonal transport

When 10 units rTNF was injected into the mouse vitreous 24 h prior to a second intravitreal injection containing only [^{3}H]proline ($n = 5$), no differences in radiolabelling of retinae or superior colliculi were found compared to 5 control mice (Fig. 1A). However, when 10 Units rTNF was intravitreally injected simultaneously with [^{3}H]proline, the amount of radiolabelled protein at the superior colliculi of these mice ($n = 7$) was 113% greater ($P < 0.01$) than in controls ($n = 7$) which received heat-inactivated rTNF together with the label (Fig. 1B, right). There were, on the other hand, no differences between [^{3}H]proline labelling in retinae from rTNF-treated eyes and heat-inactivated rTNF controls (Fig. 1B, left). Thus, a single dose of intravitreally injected rTNF caused an increase in the amount of axonally transported protein without causing a change in label incorporation at the retina. The label distribution in retinae and superior colliculi fol-

TABLE 1

INCREASE IN AXONALLY TRANSPORTED [^{3}H]PROLINE-LABELLED PROTEINS AT THE SUPERIOR COLLICULUS AS A FUNCTION OF rTNF DOSE

[^{3}H]Proline was injected into mouse vitreous simultaneously with either rTNF or a corresponding amount of heat-inactivated rTNF (HI-TNF) and tissues dissected 18 h later. Tissues were processed for liquid scintillation counting as described in Materials and Methods. Results represent mean specific radioactivity ± S.E.M. in superior colliculi and numbers of animals are shown in parenthesis. Statistical differences were determined using Student's *t*-test.

rTNF dose	dpm/mg dry tissue × 10^3		% Increase
	rTNF	HI-TNF	
0.5	2.05 ± 0.36 (5)	1.44 ± 0.32 (5)	42
1.0	1.92 ± 0.22 (6) *	1.27 ± 0.24 (6)	51
5.0	2.20 ± 0.47 (5) *	1.33 ± 0.29 (5)	65
7.5	2.72 ± 0.42 (6) *	1.50 ± 0.25 (6)	81
10.0	3.04 ± 0.49 (7) **	1.43 ± 0.20 (7)	113

* $P < 0.05$; ** $P < 0.01$.

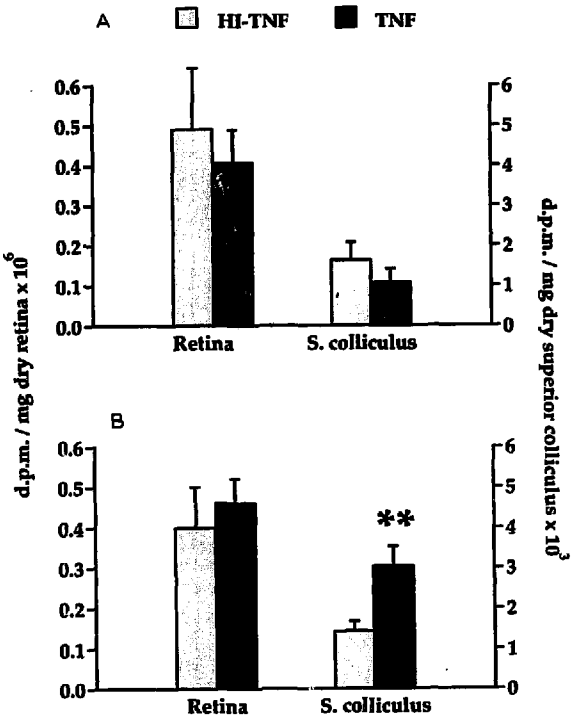

Fig. 1. [^{3}H]Proline incorporation in the mouse retina and subsequent axonal transport of radio-labelled protein to optic nerve endings at the superior colliculus. A: effect of 10 units rTNF or heat-inactivated rTNF (HI-TNF) intravitreally injected 24 h before radiolabel injection. B: effect of 10 Units rTNF or HI-TNF injected simultaneously with radiolabel. Tissues were dissected 18 h after administration of radiolabel and processed for liquid scintillation counting as described in Materials and Methods. Results represent mean ± S.E.M. Statistical differences were determined using Student's *t*-test.

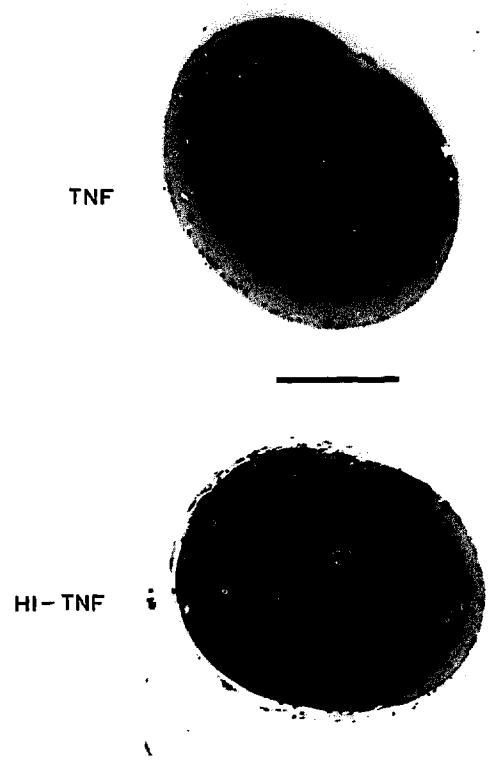

Fig. 2. Light micrographs of 1.5-μm transverse optic nerve sections taken from mice 10 days after a single intraocular injection of either 10 Units of rTNF or 10 units heat-inactivated TNF (HI-TNF). Nerves were osmium stained, processed into wax, and counterstained with toluidine blue as described in Materials and Methods. Calibration bar = 100 μm. Note that the optic nerve section taken from the rTNF-injected eye has a pale mottled appearance in the peripheral portions of the nerve trunk.

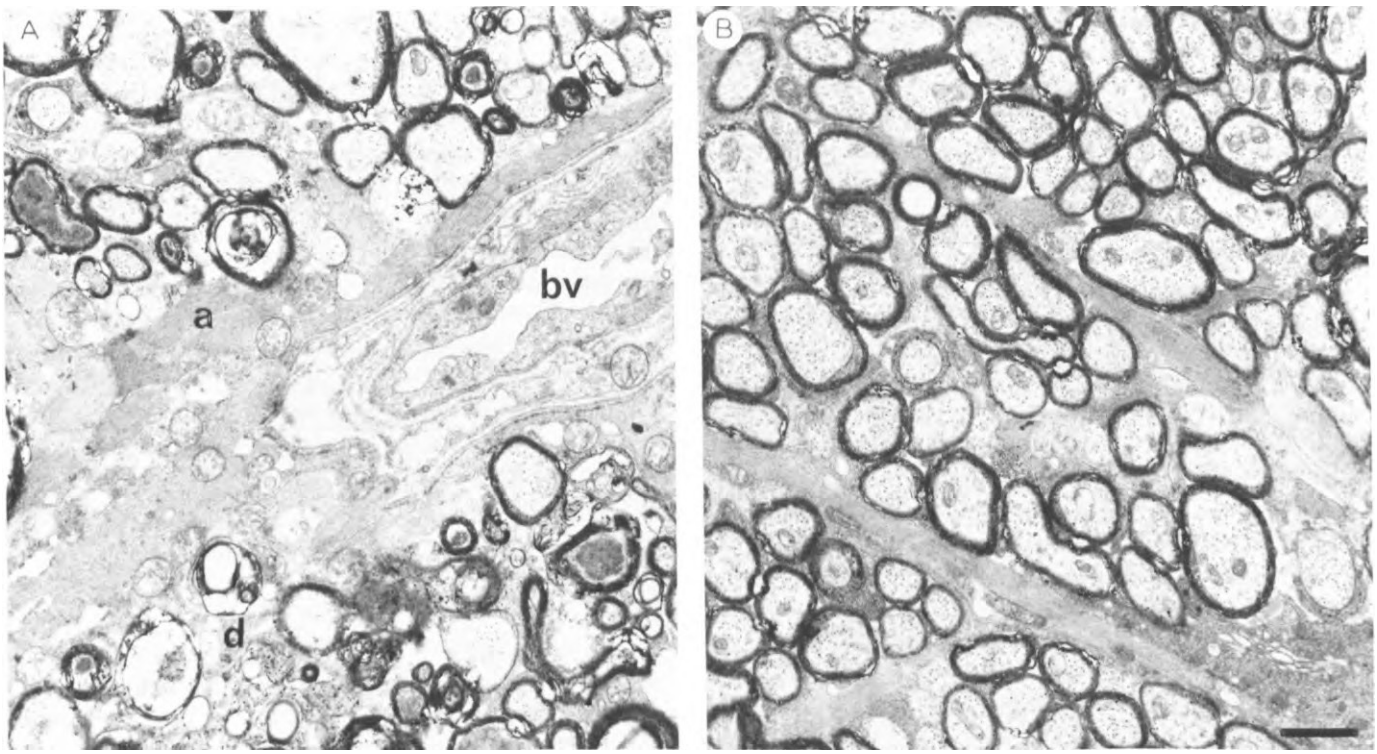

Fig. 3. A: an electron micrograph (transverse section) through an optic nerve trunk of a mouse which received an intravitreal injection of rTNF; and B: another taken from an optic nerve of a mouse eye which received intravitreal heat-inactivated rTNF. Note extensive astrocytic processes (a) close to a blood vessel (bv) and destruction of myelin (d). Calibration bar = 1 μm.

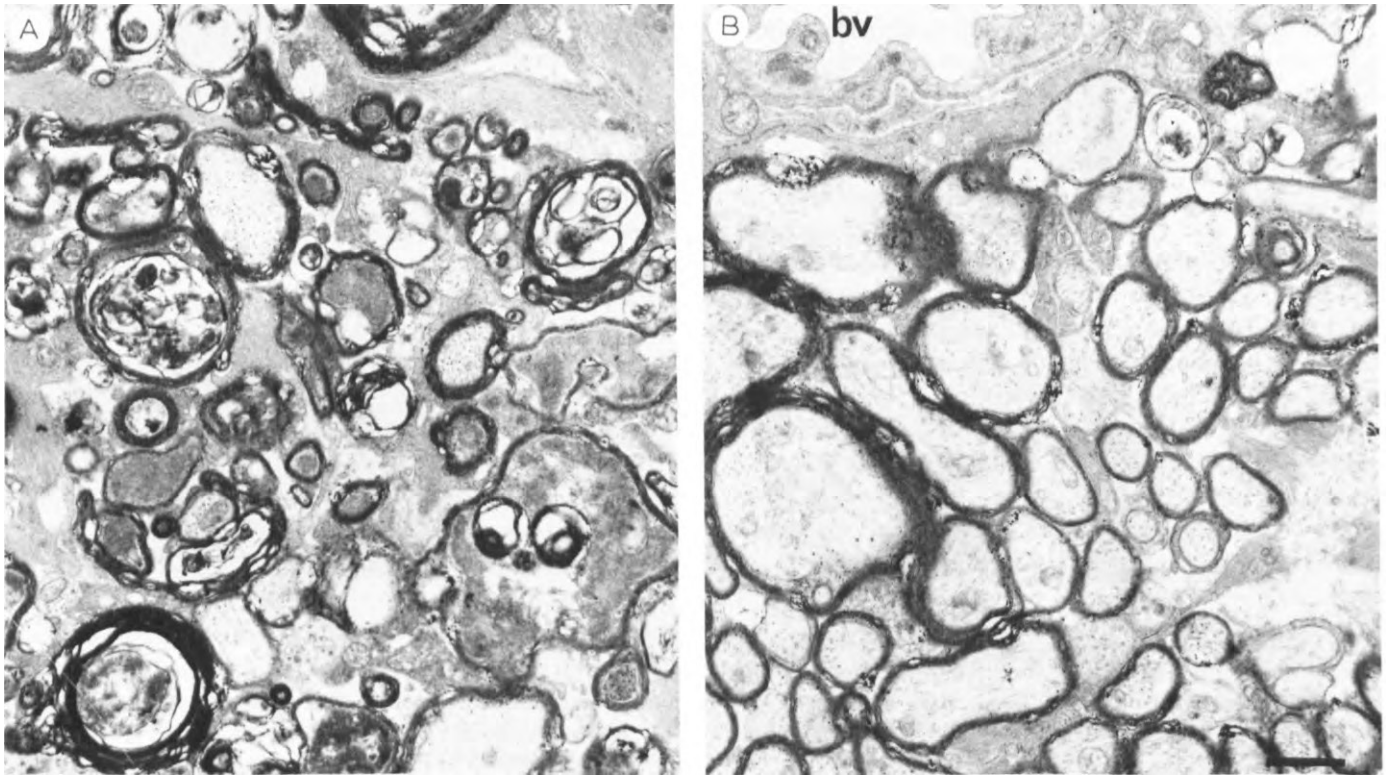

Fig. 4. A: an electron micrograph showing severe destruction of myelin as well as axonal damage in one region of an optic nerve taken from an rTNF-injected eye; and B: another taken from a different region of the same nerve showing myelinated axons of normal appearance together with minor myelin destruction close to a blood vessel (bv). Calibration bar = 1 μm.

lowing intravitreal rTNF was similar to that seen in immuno-competent SFV-infected mice several days prior to optic nerve demyelination (Tansey and Ikeda 1986; Jenkins at al. 1988). The effect of rTNF on axonal transport was found to be dose-dependent as Table 1 shows. The greater the dose of rTNF, the greater the percentage increase in the amount of radiolabelled protein at the superior colliculus.

Effect of intraocular TNF on histopathology of optic nerves

Fig. 2 shows a typical light micrograph of an optic nerve section obtained from a mouse which received a single intravitreal injection of rTNF (10 units), compared to its control which received heat-inactivated rTNF. The nerve from the rTNF-injected eye has a pale halo-like appearance and contains a region which is mottled and paler still, indicating demyelinating lesions, whilst the control nerve section obtained from the eye which received heat-inactivated rTNF shows no mottled regions. Approximately 70% of the optic nerve sections from the r-TNF-injected eyes (15–20 sections from each of the 3 nerves) showed a mottled appearance (as shown in Fig. 2) to varying degress. The lesions were focal throughout, just as those found in optic nerve sections obtained from mice infected with SFV (Tansey et al. 1985). Control nerve sections (n = 50) were, on the other hand, evenly stained and showed no sign of demyelination. It thus appears that rTNF is capable of producing demyelination similar to that following SFV infection.

In order to verify the light microscopical observation, we have examined approximately 30 electron micrographs taken from the periphery of the optic nerve trunks (where "demyelinating" lesions were frequently found) leading from rTNF-injected eyes, and 30 from a comparable region of the control optic nerve sections. Indeed, electron micrographs of the nerves from rTNF-infected eyes revealed significant pathology. An example is shown in Fig. 3 which compares an electron micrograph of an optic nerve section from a mouse which received rTNF with another from an eye which received heat-inactivated rTNF. Whilst densely packed myelinated axons and oligodendrocyte processes are seen in the control nerve (Fig. 3B), extensive fibrillar astrocytic processes and some myelin destruction near a blood vessel are apparent in the nerve from the rTNF-injected eye (Fig. 3A). To illustrate that the rTNF-induced changes are patchy and varied in their severity throughout the optic nerves, Fig. 4A shows a region of an optic nerve trunk with both myelin destruction and axonal damage, whereas another (Fig. 4B), taken from the same nerve, shows many normal myelinated axons together with some minor signs of myelin destruction close to a blood vessel.

Discussion

This study attempted to answer the question as to whether a cytokine such as TNF could be the initiator of the T-cell-mediated increase in fast axonal transport of protein and demyelination of optic nerves found in mice infected with SFV (Jenkins et al. 1988).

We found that intraocular injection of rTNF in normal mice caused both an increase in axonal transport of protein (Fig. 1; Table 1) and demyelination of optic nerve fibres (Figs. 2, 3 and 4). This could suggest that an increase in uptake and incorporation of [^{3}H]proline in the retina occurred in response to the rTNF injection. For example, histological signs of inflammatory changes have been shown in rabbit retina between 3 and 24 h after a single intravitreal rTNF injection (Brosnan et al. 1989). However, radiolabel incorporation in the retina showed no change (Fig. 1B), and the half-life of free proline in the mouse vitreous is only one hour (Nixon 1980). The increase in amounts of axonally transported protein which we found in mice after the rTNF injection must therefore have been a specific and rapid response of retinal ganglion cells. Moreover, since intravitreal injection or rTNF 24 h prior to the [^{3}H]proline injection did not result in altered labelling of either retinae or superior colliculi (Fig. 1A), whatever the structural or inflammatory changes rTNF may have caused in mouse retina, they were insufficient to promote increased axonal transport of protein. The dose-dependent effect of rTNF on the level of axonally transported protein (Fig. 2) may thus reflect a receptor-mediated response by retinal ganglion cell bodies.

The histological changes found in optic nerve sections from eyes 11 days after receiving the intravitreal injections of rTNF were similar to those found in mice 18 days after inoculation with SFV (Tansey et al. 1985; Ikeda and Tansey 1986), although rTNF caused some axonal destruction (Fig. 3). As in the SFV-infected mouse model of multiple sclerosis, lesions were frequently found near to blood vessels in the optic nerves following intravitreal rTNF injection (Fig. 4). The pattern of demyelination caused by rTNF therefore appears to be consistent with the perivascular demyelination commonly found in multiple sclerosis (Dawson 1916).

The present study thus indicates that a single intraocular rTNF injection can result in changes which mimic the effects of SFV infection on axonal transport and demyelination. This leads us to put forward a hypothesis that TNF plays an important role in the T-cell-mediated increase in fast axonal transport of protein and the subsequent demyelination found in SFV-infected mice (Jenkins et al. 1988). The following information supports this hypothesis. Firstly, TNF is released by mononuclear cells in direct response to

virus particles (Aderka et al. 1986) and it possesses anti-viral properties to both RNA and DNA virus infections in several different cell types (Wong and Geoddel 1986). Secondly, infiltration of the retina and optic nerve by inflammatory cells has been demonstrated in SFV-infected mice (Illavia et al. 1981; Pathak and Webb 1988). Thirdly, in addition to T-cell-activated macrophages, T-cells themselves release TNF (Steffen et al. 1988; Limb et al. 1989).

A number of recent studies have indicated that TNF could be involved in immune-mediated inflammatory responses and demyelination in the mammalian central nervous system (Selmaj and Raine 1988; Brosnan et al. 1989, Simmons and Willenborg 1990). This is the first in vivo study showing rTNF mediates increased axonal transport of protein in the optic nerve and, after some delay, demyelination. The consequences of TNF's action on neuronal physiology could range from the subtle to the extreme. We have suggested that intercellular communication between optic nerve axons and myelin may be disturbed due to TNF's action on retinal ganglion cells, and that this may lead to demyelination. It is also possible, moreover, that TNF is responsible for acute symptoms seen in early stages of SFV-mediated demyelinating disease or multiple sclerosis, e.g. paralysis.

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