

# Further ultrastructural studies of lesions produced in the optic nerve by Tumor necrosis factor alpha (TNF- $\alpha$ ): a comparison with experimental Creutzfeldt-Jakob disease

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**Abstract.** We report here that intraocular injection of recombinant TNF- $\alpha$  produces lesions in the optic nerve indistinguishable from those reported for the panencephalopathic type of Creutzfeldt-Jakob disease (CJD). The lesions were patchy and confined to the injected optic nerve. Axons show variable features of degenerations. Numerous vacuoles distended the myelin sheath. Hypertrophic astrocytes were numerous and many active macrophages containing digested myelin debris and lyre-like paracrystalline bodies. At high power, myelinated axons were observed as enveloped by astrocytic processes; formation of labyrinth-like network of such processes around damaged axons were observed. In conclusions, lesions produced by TNF- $\alpha$  mimic those of the panencephalopathic type of CJD, in direct support of our previous ultrastructural, immunohistochemical and molecular data on TNF- $\alpha$  involvement in CJD pathogenesis.

**Key words:** Creutzfeldt-Jakob disease, TNF- $\alpha$  , prion disorders

## INTRODUCTION

Creutzfeldt-Jakob disease (CJD) is a rare slow transmissible human dementia caused by a poorly defined infectious agent differently known as an unconventional virus, prion (hence the term "prion disorders) or virino (Liberski 1993a,b). Along with kuru, Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI) in man, natural scrapie in sheep and goats, transmissible mink encephalopathy in mink, chronic wasting disease (CWD) in captive mule deer and elk and recently discovered bovine spongiform encephalopathy (BSE). CJD is classified as a subacute spongiform virus encephalopathy and basically regarded as a poliоencephalopathy or disorder of the grey matter. Recently, however, a panencephalopathic type of CJD characterized by severe involvement of the white matter has been defined (Park et al. 1980, Mizutani et al. 1981, Vallat et al. 1983, Liberski et al. 1989).

We reported previously that vacuoles within the myelin sheath (myelin "ballooning") accompanied by an exuberant astrocytic and macrophagic reaction are the hallmark of the panencephalopathic model of CJD in mice (Liberski et al. 1990a,b, 1991). Such changes, albeit with lower frequency, were subsequently reported in experimental scrapie in hamsters and in natural BSE in cattle (Liberski et al. 1992a,b). Moreover, we suggested that TNF- $\alpha$ , a lymphokine released from activated monocyte/microglial cells and astrocytes, may be involved in the morphogenesis of myelin dilatation (Liberski et al. 1990a). As a direct test for this hypothesis, we demonstrated that TNF- $\alpha$ , following intraocular injection in mice, produced lesions in the optic nerve indistinguishable from those mentioned above in the white matter of CJD (Liberski et al. 1993). In this communication we report the detailed ultrastructural pathology of such a model and compare it with the panencephalopathic model of CJD.

## METHODS

Rules of research animal use as promulgated by the National Institutes of Health were observed

through experiments (National symposium on imperatives in research animal use, 1984). In particular, all animals were anesthetized before any procedure.

### Intraocular injection of TNF- $\alpha$

The details of this model are described elsewhere (Liberski et al. 1993). Briefly, approximately  $10^4$  units of recombinant TNF- $\alpha$  were injected into the vitreous of the right eye of ten 4- to 5-week old NIH Swiss mice (Animal Production Area, Frederick Cancer Research Facility, Frederick, MD 21701). The left eye, which served as a control, was injected with saline. Optic nerves were harvested from all animals 7 days following injection and processed for routine electron microscopy (*vide infra*). The time schedule of this experiment and the dose of TNF- $\alpha$  were chosen to obtain the maximum effect of TNF- $\alpha$  as predicted from *in vitro* studies (Selmaj et al. 1988).

### Studies of experimental Creutzfeldt-Jakob disease (CJD)

The Fujisaki strain of CJD virus, isolated from the brain of 56-year-old man with progressive dementia, was passaged three times in mice in the LCNSS (Liberski et al. 1989). The neuropathology of this case was characterized by severe destruction of the white matter.

One hundred weanling, 4- to 5-week old NIH Swiss mice in 3 different experiments were injected intracerebrally with 0.03 ml of a 10% clarified suspension prepared from mice terminally ill with the Fujisaki strain of CJD virus (titer,  $3.1 \times 10^4$  LD<sub>50</sub> by the intracerebral route). The incubation period ranged from 16 to 18 weeks. Control animals were injected with saline. Animals were randomly distributed to electron microscopic, immunohistochemical and molecular studies (Nerurkar et al. manuscript in preparation).

### Electron microscopy

Paired optic nerves from TNF- $\alpha$ -injected and saline-injected eyes were fixed in 2.5% glutaralde-

hyde and 1% paraformaldehyde in phosphate buffer, pH. 7.4. Mice inoculated intracerebrally with CJD virus and sham-inoculated control animals were killed at weekly intervals by intracardiac perfusion with 180 ml of 1% paraformaldehyde and 1.5% of glutaraldehyde prepared in phosphate buffer (pH 7.4). Perfused animals were kept at 4°C for two hours, then the brains were removed and rinsed in cold fixative overnight. Several samples (1 mm<sup>3</sup>) were dissected, rinsed in phosphate buffer, post-fixed in 1% osmium tetroxide, dehydrated through a graded series of ethanols and propylene oxide and embedded in Embed (Electron Microscopy Sciences, Ft. Washington, PA). Ultrathin sections were stained with lead citrate and uranyl acetate, and specimens were examined using EM 109 Zeiss transmission electron microscope at 80kv.

## RESULTS

Densely packed myelinated fibers separated by thin astrocytic septae comprised normal optic nerve following injection with saline. In contrast, optic nerves injected with TNF- $\alpha$  demonstrated the abundance of patchy active degradation of myelinated fibers by macrophages and astrocytes observed with a different intensity in different segments. However, TNF- $\alpha$ -induced optic nerve lesions were less pronounced than those observed at the terminal stage of CJD-infected mice (Fig. 1).

Extensive lesions in the TNF- $\alpha$ -injected optic nerve were visible at low power. These consisted of myelinated fibers in different stages of destruction, accompanied by reactive astrocytes and activated macrophages containing myelin fragments and even segments of myelinated fibers within digestive chambers (Figs. 2-4). Analogous pictures were easily identified in CJD-infected mice (Fig. 1). Some remnants of myelinated fibers were observed within astrocytes recognized because of innumerable glial filaments. Myelin "ballooning" was indistinguishable from those of the panencephalopathic model of CJD (Fig. 1) and frequently the cytoplasm of an activated macrophage was discerned in close contact with the outermost layer of myelin sheath lining the

vacuole (Fig. 2). A labyrinth of channels connected myelinated fibers undergoing destruction to the cytoplasm of an astrocyte or a macrophage, then spirally enveloped such a segment to eventually enclose it within the cytoplasm (Fig. 3). Activated macrophages and astrocytes participated in the final destruction of myelinated fibers (Fig. 4).

## DISCUSSION

Vacuolation of myelinated fibers is now known to be an important feature of the ultrastructural pathology of the panencephalopathic form of CJD (Liberski et al. 1991). Such a lesion is not specific, however. Myelin sheath dilatation ("ballooning"), nearly identical to that observed in CJD virus-infected mice, is frequently found in different models of experimental allergic encephalitis and in multiple sclerosis (Brosnan et al. 1988). By the same token, upregulation of TNF- $\alpha$  seems to be a common and probably unspecific denominator for many types of disorders affecting myelin.

The exact pathogenesis of myelin dilatation in CJD and demyelinating diseases is unknown. It has been reported that myelin sheath vacuolation, ultrastructurally indistinguishable from that presented here, had been produced in organotypic cultures treated with human recombinant TNF- $\alpha$  (Selmaj et al. 1988). Myelin ballooning was accompanied by oligodendrocyte degeneration and astrocytic hypertrophy. Moreover, cytotoxic activity of TNF- $\alpha$  and lymphotoxin (LT) was reported (Selmaj et al. 1990, 1991a,b). The hypothesis of TNF- $\alpha$  involvement in myelin destruction was further substantiated by immunohistochemical detection of TNF- $\alpha$  expression in astrocytes in CJD (Liberski et al. 1990), multiple sclerosis (Hoffman et al. 1989, Selmaj et al. 1991a), adrenoleukodystrophy (Poweres et al. 1992) and HIV-infected brains (Maier et al. 1989, Grimaldi et al. 1990), and by blocking of a passive transfer of EAE by anti-TNF $\alpha$ -neutralizing antibodies (Hauser et al. 1990, Ruddle et al. 1990, Selmaj et al. 1991b) or by TNF- $\alpha$  inhibitor, pentoxifylline (Nafaf et al. 1993). Furthermore, the injection of TNF- $\alpha$  into the vitreous of the mouse eye produced lesions in the

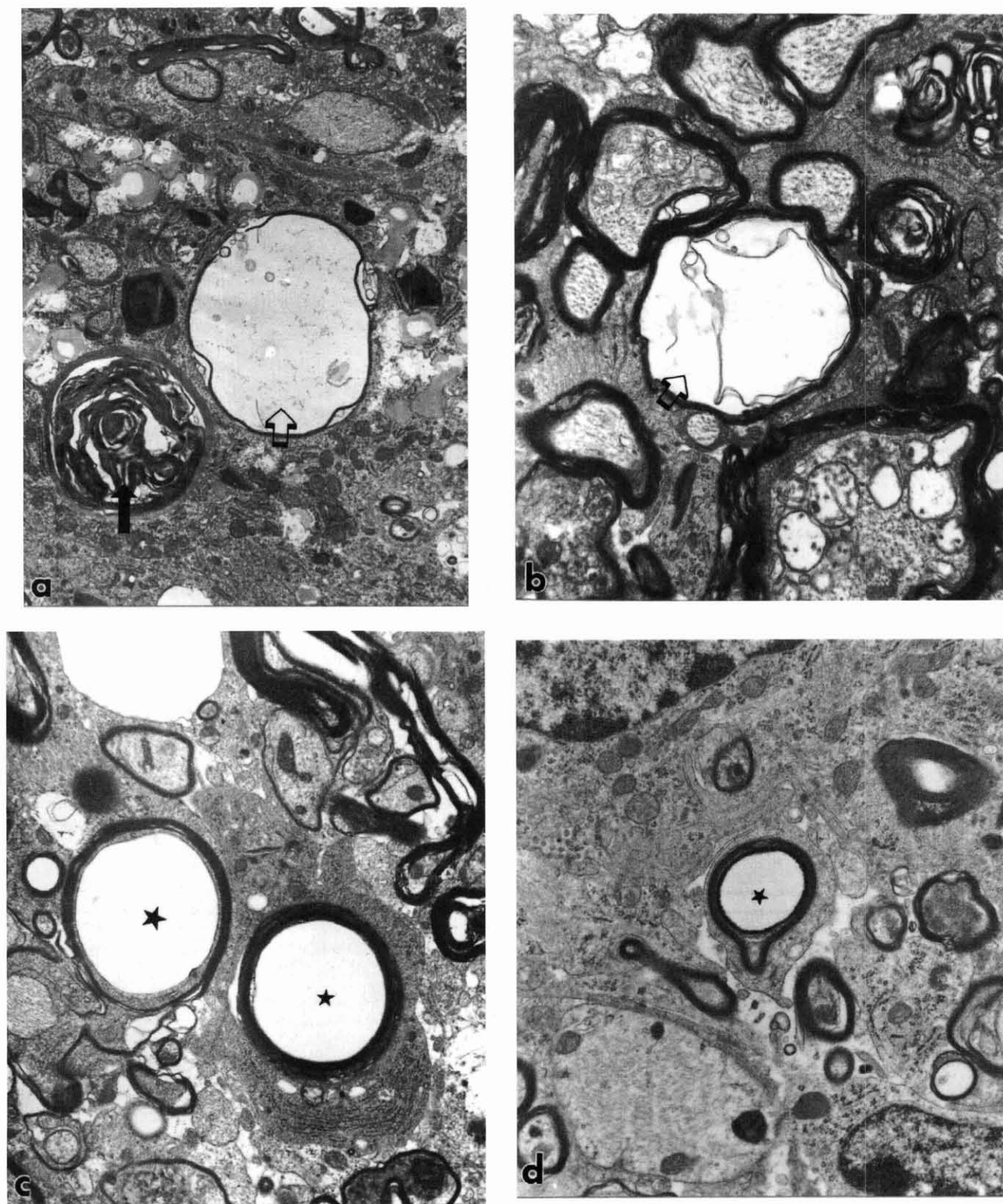


Fig. 1. A,C,D, terminal stage of CJD-infected mouse. Note intramyelin vacuole (open arrow), myelin debris (arrow) and degenerating myelinated fibers within cytoplasm of macrophages (stars); B, intramyelin vacuole in TNF- $\alpha$ -injected optic nerve (open arrow). Magnifications,  $\times 12\,000$ .

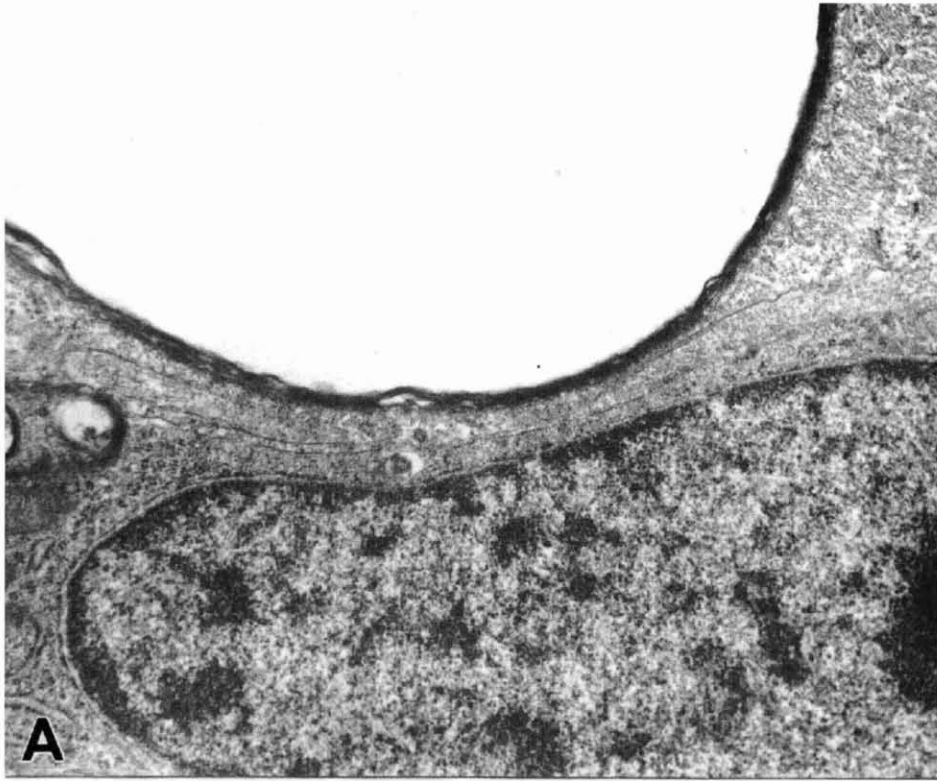


Fig. 2. A cytoplasm of an activated macrophage in close contact with the outermost layer of myelin sheath lining the vacuole of (A)  $\text{TNF-}\alpha$ -injected optic nerve and (B) CJD-infected mouse brain. Lead citrate and uranyl acetate. Magnification,  $\times 12000$ .



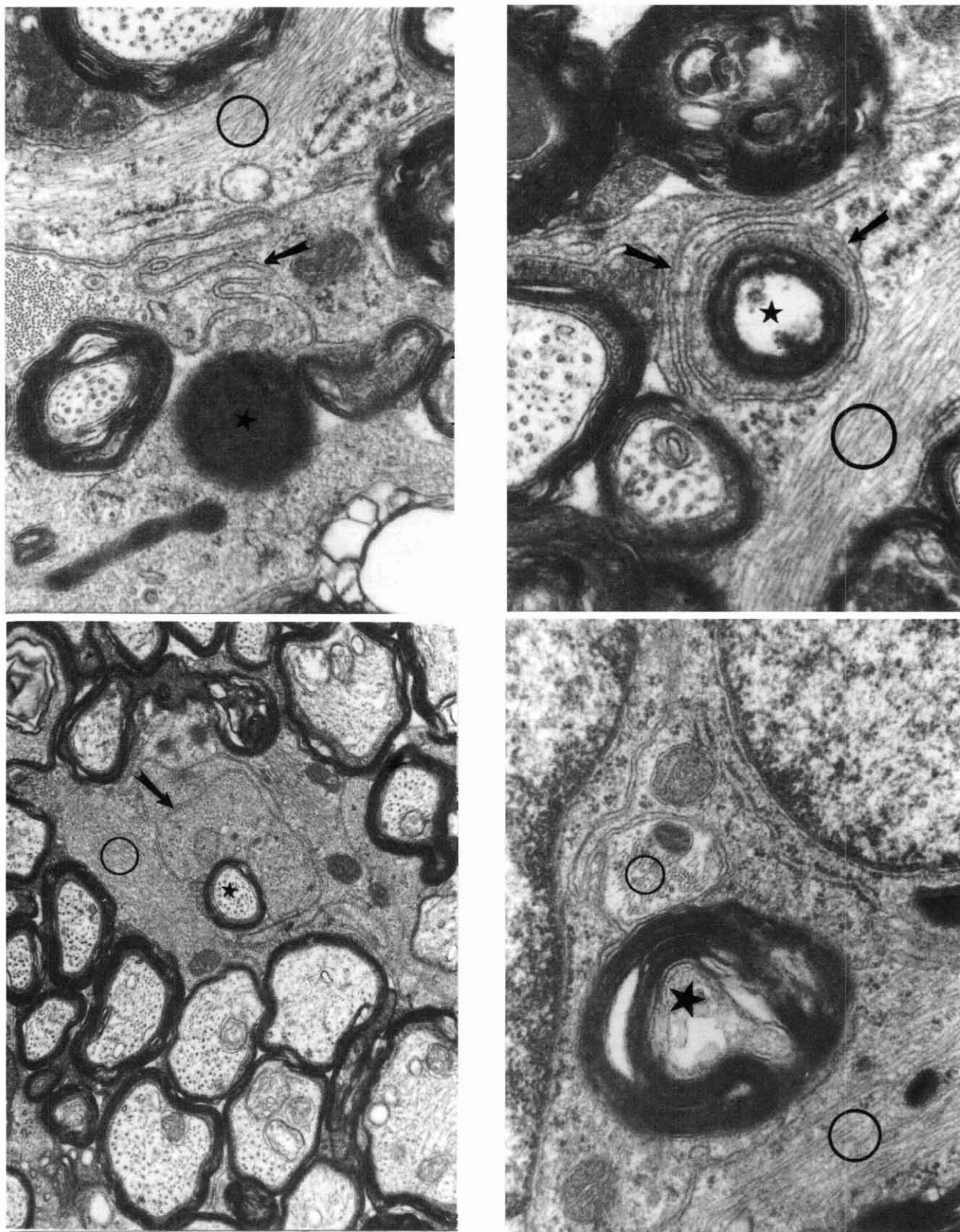


Fig. 3. Myelinated axons (stars) enveloped with glial processes (arrows) of TNF- $\alpha$ -injected optic nerve. Note numerous glial filaments (circles). Lead citrate and uranyl acetate. Magnifications, x 30 000.

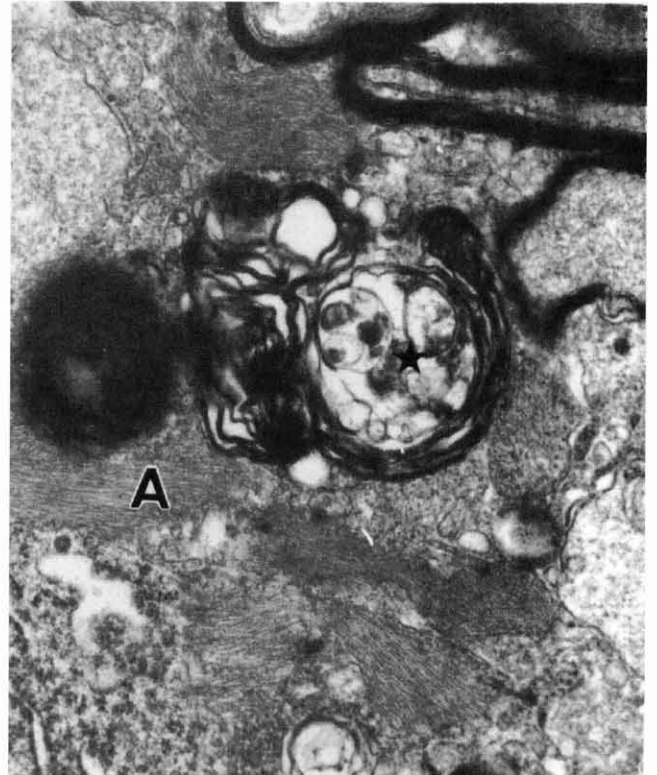
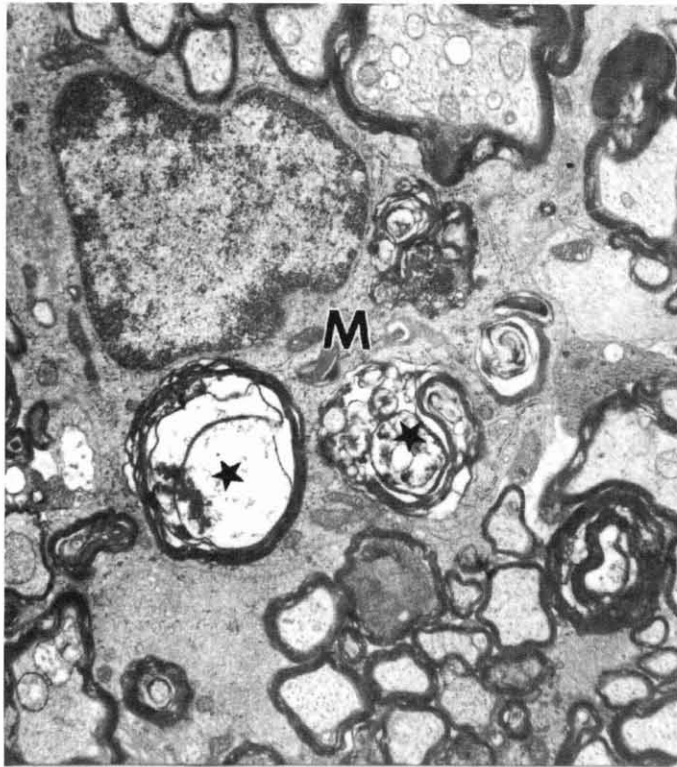
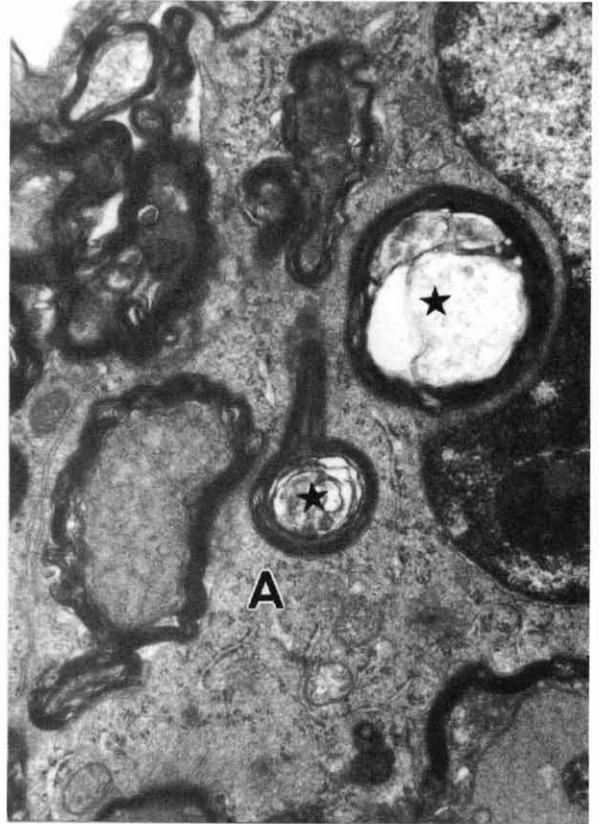
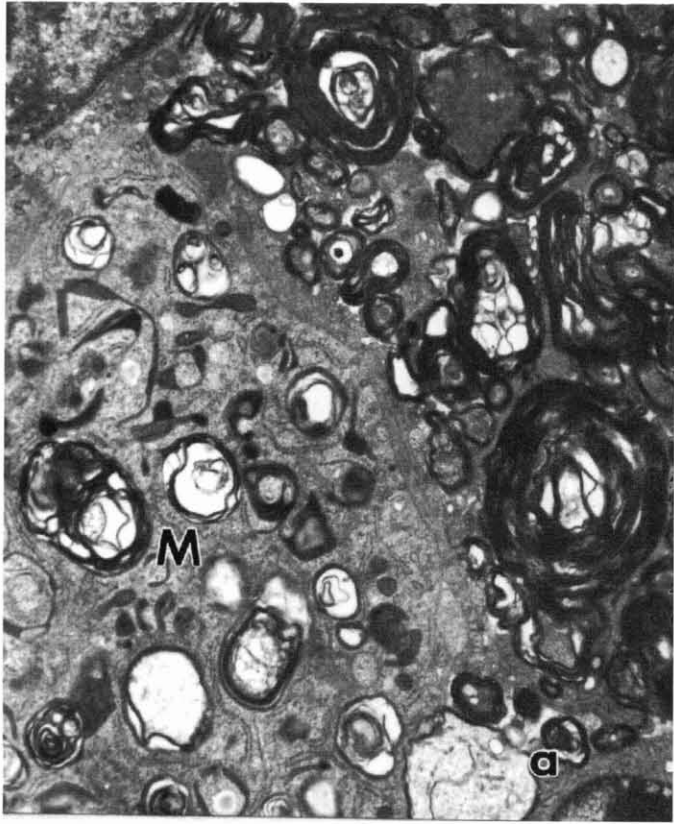


Fig. 4. Destruction of myelinated fibers by activated macrophages (M) or astrocytes (A) in TNF- $\alpha$ -injected optic nerve. Note myelin debris undergoing digestion (stars).

optic nerve indistinguishable from those observed in the panencephalopathic type of CJD (Jenkins and Ikeda 1992, Liberski et al. 1993). The ultrastructural pathology of myelinated fibers and the detection of TNF- $\alpha$  in hypertrophic astrocytes in areas of myelin vacuolation in mice infected with the Fujisaki strain of CJD virus, indicate that myelin vacuolation in the subacute spongiform virus encephalopathies (Liberski et al. 1989b, 1990a), not unlike that in other disorders of myelin (Brosnan et al. 1988), may be a cytokine-mediated phenomenon.

CJD and the other subacute spongiform virus encephalopathies are regarded as polioencephalopathies or disorders of the grey matter. The Fujisaki strain of CJD virus is atypical in that it produces, in addition to common CJD pathology, widespread myelin and axonal damage. However, myelin pallor and focal accumulation of the products of myelin degradation are constant features of experimental kuru, CJD, scrapie and BSE but to a lesser degree (Liberski et al. 1992a,b). We thus believe that there is a common mechanism for axonal and myelin pathology in all the subacute spongiform virus encephalopathies, and TNF- $\alpha$  may be the first identifiable mediator.

How TNF- $\alpha$  actually produces the myelin damage is unknown at the present time. TNF- $\alpha$  is secreted from both microglia and astrocytes (Robins et al. 1987, Lieberman et al. 1989, Sawada et al. 1989, Chung and Benveniste 1990, Hetier et al. 1990, Chung et al. 1991, Lee et al. 1993) and one class of high affinity receptor for TNF- $\alpha$  is expressed on astrocytes (Bethea et al. 1990). Furthermore, TNF- $\alpha$  is a mitotic signal for astrocytes (Barna et al. 1990, Selmaj et al. 1991). Of note, it seems that the TNF- $\alpha$  secretion from CNS microglia/macrophages is developmentally regulated and may be even partially suppressed during different stages of macrophage maturation (Sebire et al. 1993). Astrocytes function as antigen presenting cells (APC) in the brain (Fontana et al. 1984, Frei and Fontana 1989) and this function is restricted by the expression of class II major histocompatibility complex (MHC) antigens (Lavi et al. 1988, Ben-

veniste et al. 1989). Indeed, expression of the class II MHC antigens is upregulated by the rTNF- $\alpha$  and this effect is completely blocked by anti-TNF- antibodies (Bethea et al. 1990). This enhancing effect is mediated through an "inductive signal" from IFN- $\gamma$  which increases the number of receptor sites for TNF- on astrocytes (Benveniste et al. 1989). The kinetics of induction of class II MHC mRNA by IFN- $\gamma$  and INF- $\gamma$ /TNF- $\alpha$  and the fact that this class II MHC mRNA induction is abolished by cycloheximide, a potent inhibitor of protein synthesis, suggests that this cytokine signal is complex and involves several intermediate steps requiring *de novo* protein synthesis (Vidovic et al. 1990). Interestingly, selected neuronal populations also express TNF- $\alpha$  following neuronal transplants (Tchelingerian et al. 1993). Thus neurons may be directly implicated in a cytokine network.

CJD and other subacute spongiform virus encephalopathies, are characterized by the absence of any detectable immune response (Kingsbury et al. 1981). By the same token, the functional status of the lymphoreticular system profoundly influences the pathogenesis of diseases but only following peripheral inoculation (Liberski 1993a,b). It is however completely conceivable that TNF- $\alpha$  secreted from activated astrocytes participates in an abortive immunological reaction toward the CJD virus. Furthermore, TNF- $\alpha$  acting in an autocrine manner through the high affinity receptor expressed on astrocytes may contribute to the development of astrocytic gliosis, one of the neuropathological hallmarks of CJD. Regardless its role in CJD, TNF- $\alpha$  warrants further studies.

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