

Demyelination in canine distemper encephalomyelitis: an ultrastructural analysis

BRIAN A. SUMMERS¹ and MAX J. G. APPEL²

Departments of ¹Pathology and ²Microbiology (James A. Baker Institute), New York State College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA

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Summary

A morphological study of selected white matter lesions was carried out in three dogs with canine distemper encephalomyelitis. Two dogs had experimental infections while the third was a spontaneous case. Two stages were identified in the process of demyelination. The earliest evidence of myelin injury was a ballooning change in myelin sheaths involving single or multiple axons. This was followed by a progressive stripping of compact sheaths by the cytoplasmic fingers of phagocytic cells which infiltrated and removed myelin lamellae. Some axonal necrosis also accompanied these changes. Where demyelination occurred, canine distemper viral nucleocapsids were found in astrocytes, macrophages, ependymal cells and infiltrating lymphocytes. In contrast, oligodendrocytes were conspicuous by their apparent lack of infection. Thus it seems that myelin loss cannot be ascribed to oligodendrocyte infection. Perturbed astrocyte function following canine distemper viral infection may cause oedema of myelin sheaths, leading to ballooning and primary demyelination. Cells which phagocytosed myelin were mainly identified as microglial cells with lesser involvement by astrocytes. Rarely, oligodendrocytes also acted as macrophages. Myelin debris was engulfed in bulk or as small droplets into coated pits. Remyelination was present in established plaques although not in great abundance, perhaps due to the diminished oligodendrocyte numbers and a relative increase in immature forms of these cells. These observations are compared to similar changes observed in other demyelinating diseases of animals and man.

Introduction

Of the human demyelinating diseases, multiple sclerosis (MS) remains the most important with perhaps 250 000 cases in the United States alone (Waksman, 1981). The aetiology and pathogenesis of this disorder remain elusive but are areas of active investigation. Studies of murine models of MS have demonstrated that myelin loss may follow lytic oligodendrocyte infection (mouse corona virus infection, see Lampert *et al.*, 1973) or can result from an immunopathological attack on persistently infected oligodendrocytes (Theilers disease, see Rodriguez *et al.*, 1983). Canine distemper encephalomyelitis (CDE) has been proposed as a further model of MS (Koestner, 1975). Canine distemper is caused by a morbillivirus, certain strains of which will induce a high incidence of CNS demyelination (Summers *et al.*, 1979; Higgins *et al.*, 1982b).

Although several authors have reported on the ultrastructural changes in spontaneous (Wisniewski *et al.*, 1972; Raine, 1976) and experimental (McCullough *et al.*, 1974; Higgins *et al.*, 1982b) CDE, the mechanism of demyelination remains unclear. Previous studies have documented the areas of predelic-

tion within the CNS for demyelinating lesions to develop, and their chronological appearance (McCullough *et al.*, 1974; Summers *et al.*, 1979). Given this background information, we have performed a detailed electron microscopic study of selected areas of the neuraxis in an attempt to clarify the pathogenesis of demyelination in this viral encephalomyelitis of the dog.

Materials and methods

The CNS tissues studied were derived from three dogs – two experimental and one spontaneous case of CDE. The former resulted from intranasal infection of specific-pathogen-free Beagle dogs with the Cornell A75-17 strain of canine distemper virus (CDV). These dogs were perfused at 24 and 45 days post-infection with 3–4 litres of 1% Karnovsky's solution delivered into the left ventricle. Specimens were taken from periventricular white matter areas: the crus of the fornix at the lateral ventricle and the cerebellar medulla adjacent to the fourth ventricle. The third animal (spontaneous case) had shown neurological signs for 4 weeks. Following perfusion of this animal, tissue was taken from the ventral funiculus of the lumbar spinal

cord. Tissue from this dog was kindly provided by Dr C. S. Raine, New York. From the experimental cases, adjacent areas of the brain were postfixed in 10% buffered formalin and processed through paraffin for light microscopy. Sections were stained with haematoxylin and eosin. Tissues for electron microscopy were routinely postfixed in 1% OsO₄, dehydrated and embedded in Epon. Sections, 1 µm thick, stained with toluidine blue were examined for white matter lesions. Selected areas were thin sectioned, stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope.

Results

Light microscopy

In paraffin sections from the dog killed at 24 days post-infection, myelin injury was first seen as areas of sponginess or vacuolation, often below the ependyma or pia mater. As this spongiform change became more severe, vacuoles apparently coalesced, producing a zone (or plaque) of demyelination (Fig. 1). Very rarely it was possible to visualize the axon within the distended myelin sheaths (Fig. 2). This myelin change was consistently accompanied by a pronounced astrocytosis; some reactive astrocytes had hypertrophic, open-faced nuclei and swollen cytoplasm (Fig. 3). Multi-nucleated astrocytes were also seen. A mild proliferation of rod cells (microglia) accompanied this astrocytosis. Expansion of white matter lesions appeared to develop by further ballooning change at the margin of a zone of demyelinated white matter (see Fig. 1); a few distinct vacuoles could usually be found within the demyelinated zone as well. As lesions developed an inflammatory nature (seen at day 42 and in the spontaneous case) with an influx of lymphocytes and plasma cells, the demyelinated neuropil became more rarified, disrupted and in areas frankly necrotic if the mononuclear cell infiltrate was heavy. Foamy macrophages were evident in the meshwork of naked axons and glial processes.

In plastic sections, 1 µm thick, greater detail of the ballooning myelin change could be appreciated. Furthermore, individual demyelinated axons could be confidently identified (Fig. 4). Initially the Vir-

chow-Robin spaces were empty, apart from rare flattened perivascular cells, best appreciated in plastic sections. As the lesions become inflammatory it was evident that the perivascular cuffs were often located at the margins of the plaques (Fig. 5) and not necessarily related to plaque expansion.

Electron microscopy

In areas of otherwise normal white matter, severely ballooned myelin sheaths, derived from a single axon, could be found (Fig. 6). The affected axon sometimes had a misshapen or distorted profile, as if compressed, but filament accumulation within the fibre was usually lacking. The ballooned myelin sheath was formed from full or partial thickness of the sheath. It was not possible to determine whether sheaths split consistently at the major dense line or intraperiod line. Ballooned sheaths were empty or contained a slightly flocculent, electron-dense material. Sometimes processes of astroglial or microglial cells were seen adjacent to the swollen sheath. (Because of the controversy regarding microglial cells – see Kitamura *et al.* (1984) and Schelper & Adrian (1986) – which is discussed below, we have used this term to denote the precursors of the predominant macrophages within the plaques.) In other areas, myelin sheath ballooning was seen about groups of several axons. Whereas some fibres appeared intact, others were swollen with the accumulation of lysosomal and membranous dense bodies, mitochondria and multivesicular bodies. These degenerate, sometimes swollen axons were also partially or totally denuded of myelin (Fig. 7). Axonal necrosis and lysis was evident from large, empty myelin sheaths and from broad expanses of astroglial and microglial cell processes, apparently filling the neuropil where destruction of myelinated fibres had occurred.

Neighbouring these areas of ballooning myelin change were foci of primary demyelination. Such areas contained virus-infected cells of several types. Canine distemper viral nucleocapsids were observed within macrophages and ependymal cells (Fig. 8) and within astrocytes and infiltrating lymphocytes. Infection of all these cells has been well documented in

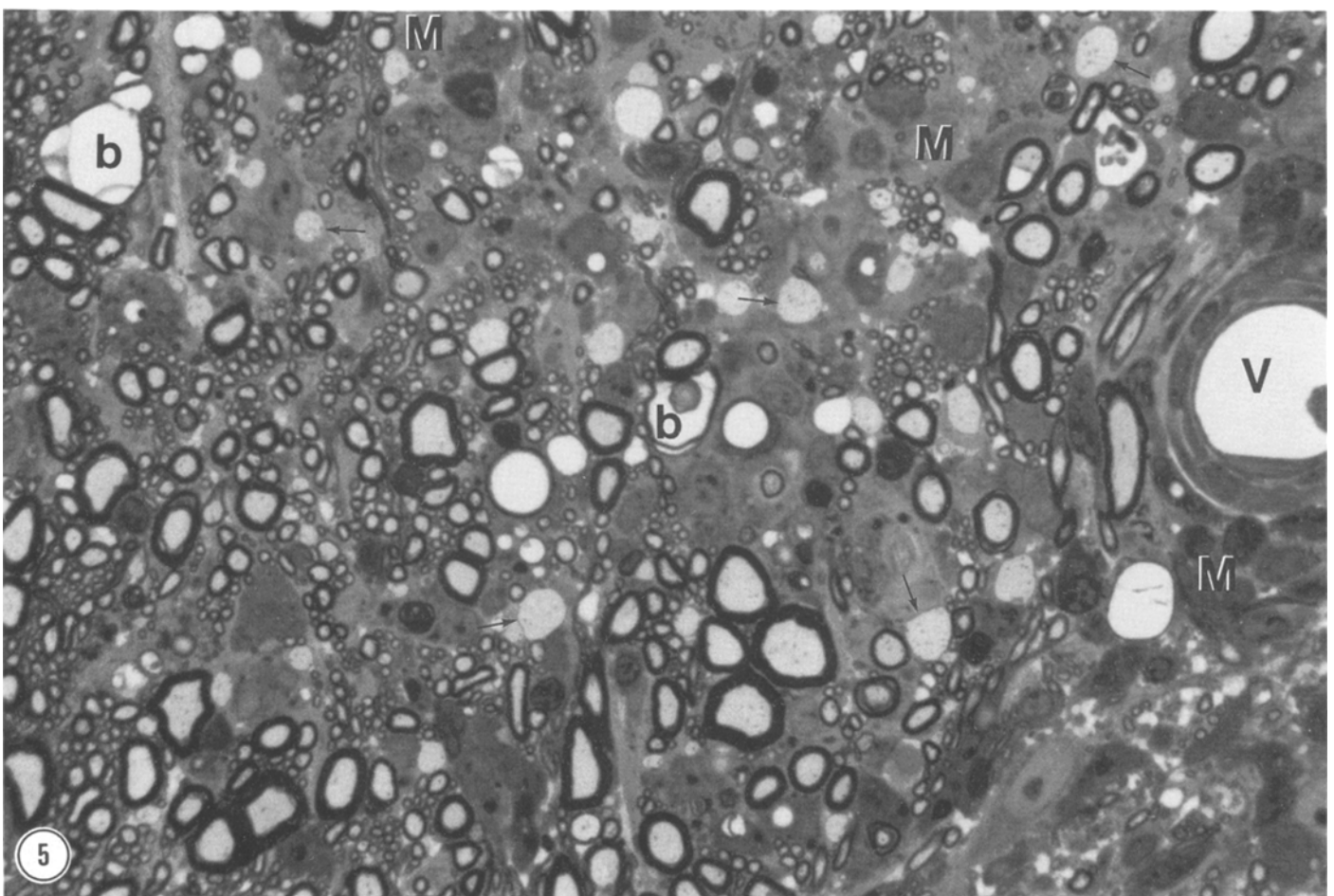
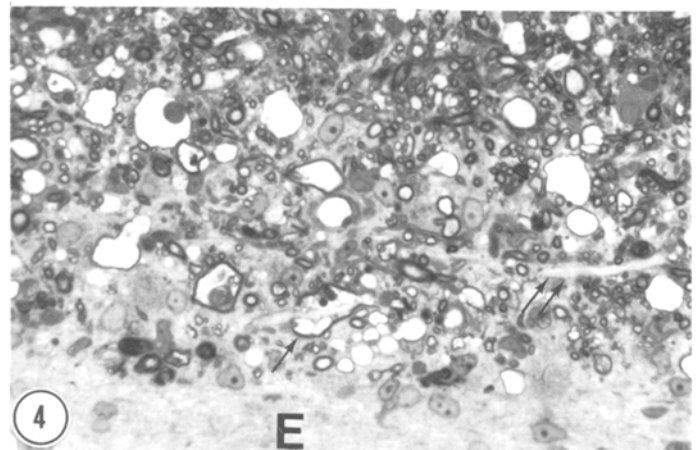
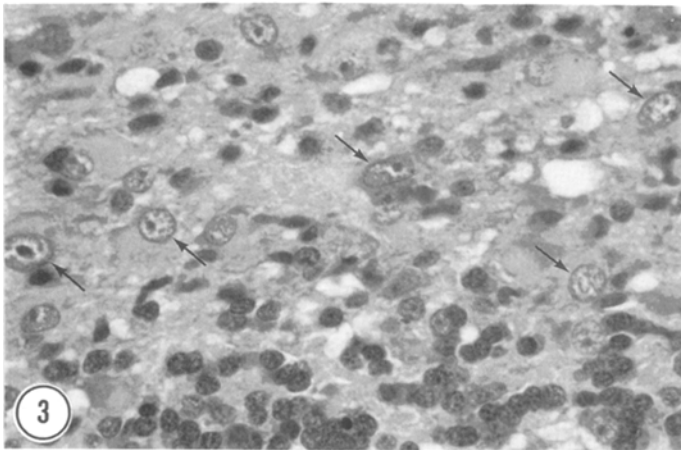
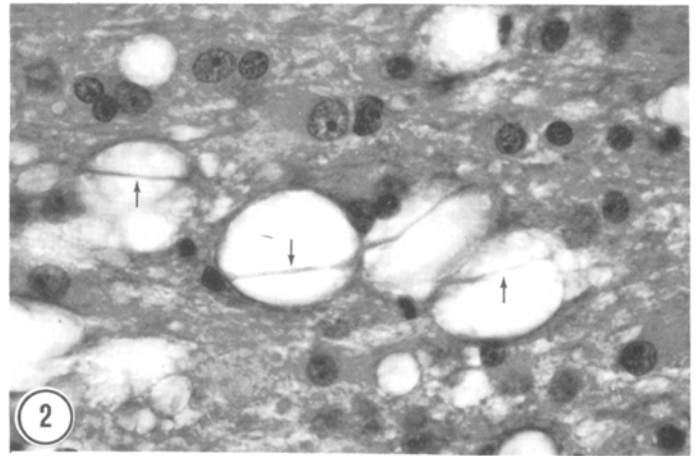
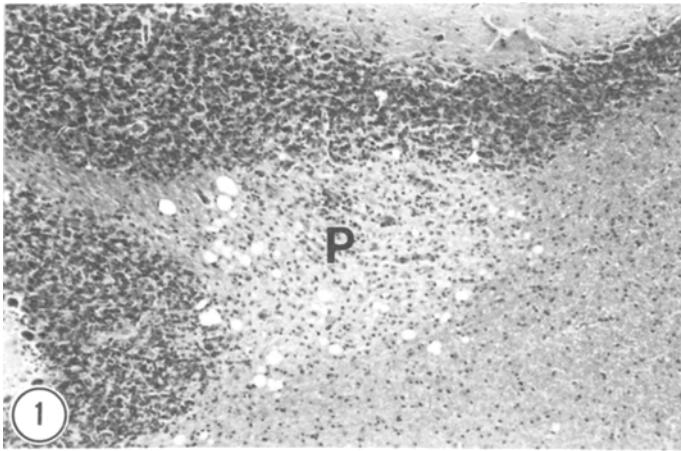
Fig. 1. Cerebellum showing pale, mildly gliotic demyelinated plaque (P), myelin vacuolation at the margins and normal white matter. H. and E. × 150.

Fig. 2. Axons (arrows) can be seen traversing ballooned myelin sheaths. H. and E. × 480.

Fig. 3. Hypertrophic astrocytes (arrows) in white matter adjacent to granule cells of cerebellar cortex. H. and E. × 480.

Fig. 4. Early white matter lesion adjacent to ependymal cells (E). Note prominent myelin vesiculation. One axon shows segmental demyelination with ballooning of the adjacent internode (single arrow). Another axon is fully demyelinated (double arrows). Plastic section, 1 µm thick. × 480.

Fig. 5. Inflammatory white matter lesion. A perivascular cuff (blood vessel V) is seen at the edge of a plaque. The lesion contains a few ballooned sheaths (b), several demyelinated axons (arrows) and a light diffuse infiltrate of mononuclear inflammatory cells (M). Plastic section, 1 µm thick. × 1280.



previous studies (Wisniewski *et al.*, 1972; McCullough *et al.*, 1974; Summers *et al.*, 1978, 1979, 1984; Higgins *et al.*, 1982b). In contrast, however, viral nucleocapsids were conspicuously lacking in oligodendrocytes, being seen in but a single light oligodendrocyte.

Areas of demyelination contained increased numbers of astrocytes and microglial cells. Reactive fibrous astrocytes (Fig. 9) were identified by their ovoid nuclei with dispersed chromatin, pale cytoplasm and conspicuous bundles of intermediate filaments. Hypertrophic astrocytes were cells with more extensive cytoplasm which contained many microtubules but few filaments, several lysosomes and free ribosomes in the perikaryon. Some hypertrophic astrocytes had phagocytosed loops of myelin (Fig. 10) or contained digestion products thereof.

In contrast to astrocytes, microglial cells were more electron-dense with clumped heterochromatin, a more granular cytosol and characteristic long profiles of rough endoplasmic reticulum (Fig. 11). Tubuloreticular inclusions (see Fig. 6), which characteristically appear within the rough endoplasmic reticulum in CDE and other diseases (Grimley & Schaff, 1976) were a very useful marker for microglial cells; they were also observed in endothelia and pericytes.

Loss of compact myelin apparently began by the spiral wrapping of cytoplasmic processes of macrophages around intact fibres. These processes then penetrated the sheath, resulting in a concentric stripping of myelin lamellae away from the axon. Such processes could be observed within the sheath (Fig. 12) and very rarely the complete histiocyte came to occupy an adaxonal position. Stripping only occurred of compact myelin and was never seen where myelin ballooning had occurred. Separated myelin fragments were found as free, extracellular droplets or within the cytoplasm of the myelin stripping cells – which most often had the features of microglia. Occasionally, small myelin fragments were observed within coated pits (Fig. 12) on the surface of macrophages. It was very common to observe individual, fully demyelinated axons adjacent to a group of normal (myelinated) fibres. On rare occasions where nerve fibres were sectioned in longitudinal profile, segmental demyelination was observed.

Axons which had been demyelinated were often enclosed by the broad expanse of macrophage processes (Fig. 13). It appeared that these processes then retracted, leaving naked axons which floated free in the neuropil or were loosely ensheathed by the processes of fibrous astrocytes (Fig. 14).

Three types of cells were involved in myelin phagocytosis. Most were microglia (Figs 11–13) which most often appeared to perform the myelin stripping; less frequently, astrocytes contained myelin fragments. On very rare occasions, myelin loops were

found within the cytoplasm of oligodendrocytes (Fig. 15).

As plaques of demyelination expanded, numbers of oligodendrocytes within the demyelinated zone were reduced compared to areas of adjoining white matter. Rare necrotic cells were seen within the plaque, but could not specifically be identified. Oligodendrocytes could readily be found at the plaque margin and were conspicuous by the frequent presence of centrioles, perhaps indicative of mitotic activity (Raine *et al.*, 1981). Centrioles, however, were observed in other cells, albeit less frequently (mostly macrophages). In well-developed lesions, many remaining oligodendrocytes were of the light variety (Mori & Leblond, 1970). In contrast, medium and dark oligodendrocytes predominated in white matter with early lesions and in normal white matter.

The process of demyelination in CDE evolves from a non-inflammatory to an inflammatory state (McCullough *et al.*, 1974; Summers *et al.*, 1979). Tissue examined from the dog studied 24 days post-infection generally lacked perivascular mononuclear inflammatory cells. Occasional reactive macrophage-like cells, possibly derived from vascular pericytes, were found about some capillaries (Fig. 16); some of these cells contained tubuloreticular inclusions. In the 42-day dog and the spontaneous case, monocytes, lymphocytes and plasma cells from the perivascular compartment percolated into the lesion areas (Fig. 17), but were not clearly associated with expansion of the zone of demyelination (see Fig. 5).

Remyelination was only a sporadic observation and was identified as inappropriately thin sheaths unattended by myelin stripping or the presence of myelin phagocytosis. Rare findings were aberrantly located paranodal loops and tapering of the myelin sheath.

Discussion

In this study of demyelination in CDE we have focused on limited areas of the neuraxis which were defined, in previous investigations (McCullough *et al.*, 1974; Summers *et al.*, 1979), as harbouring injured myelin consistently. Light microscopic examination showed that ballooning was the earliest evidence of myelin disruption. As the plaques expanded it was possible to identify a centrally demyelinated core, a narrow vacuolated zone at the margin and the adjacent normal white matter. At the electron microscopic level, this sponginess was found to result from pronounced ballooning of myelin sheaths from the axon. Sometimes the balloon myelin resulted from a split in the myelin sheath, leaving part of the sheath still covering the axon while the remainder formed the balloon. Myelin preservation was inadequate to determine whether sheaths split at the

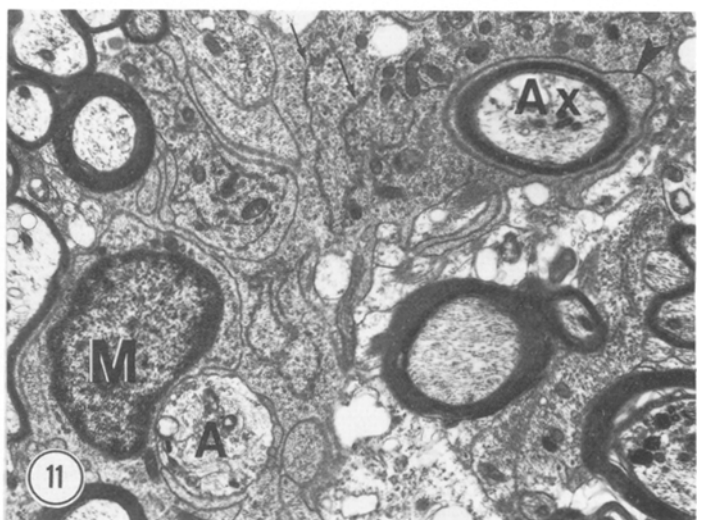
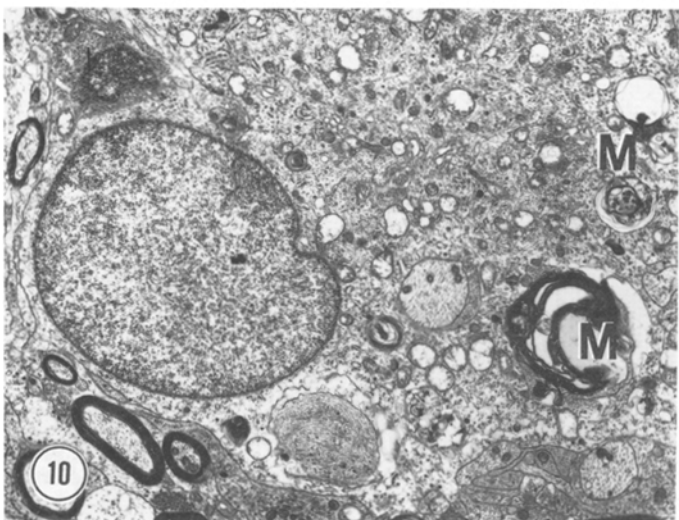
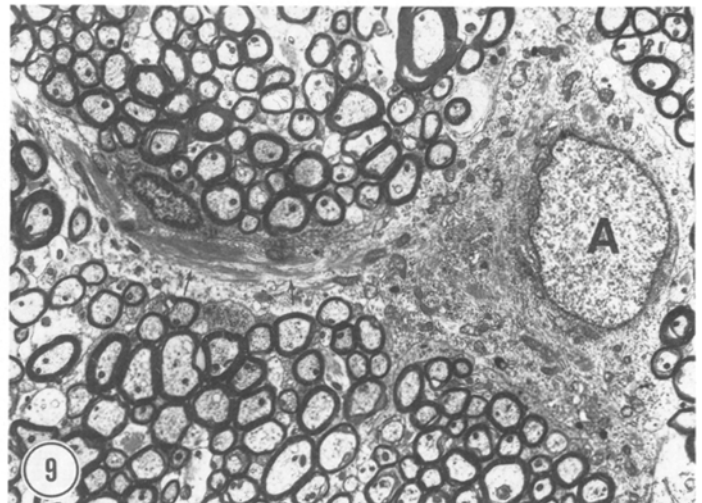
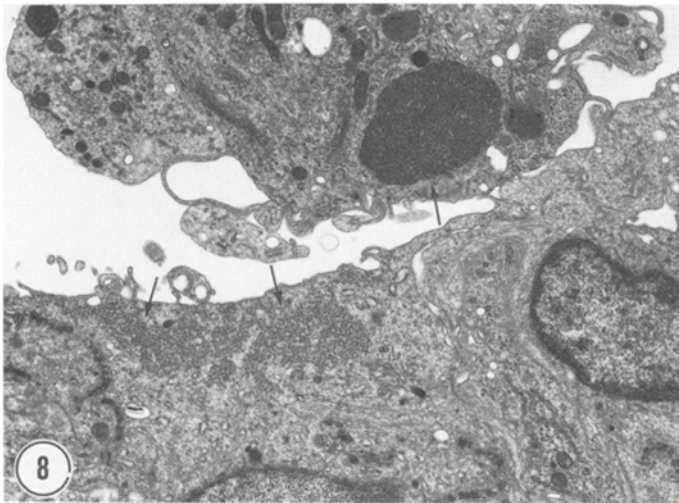
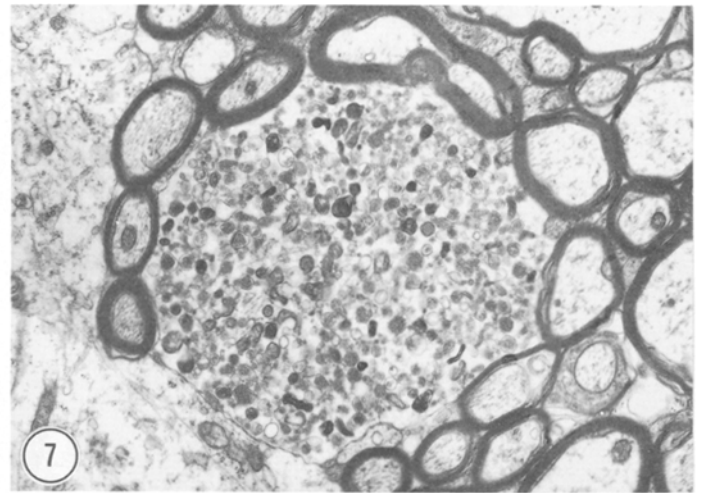
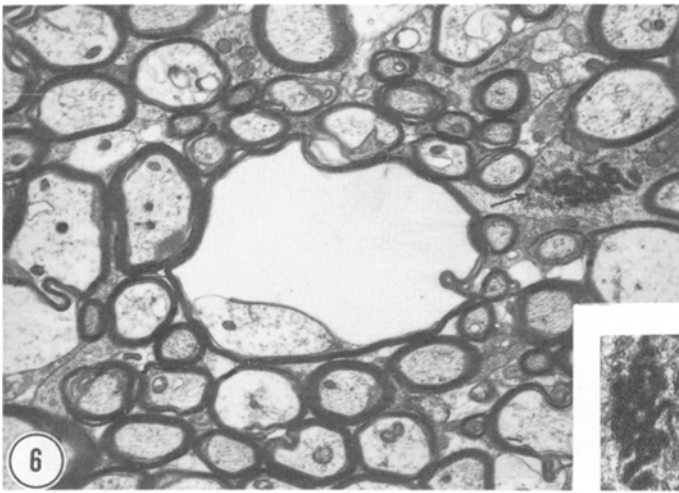


Fig. 6. Ballooning of myelin from a single axon. Other myelinated fibres are intact. Microglial cell process, containing a dense tubuloreticular inclusion (arrow), abuts the ballooned sheath. $\times 6300$. Inset shows the tubuloreticular inclusion contiguous with the endoplasmic reticulum (arrow). $\times 8820$.

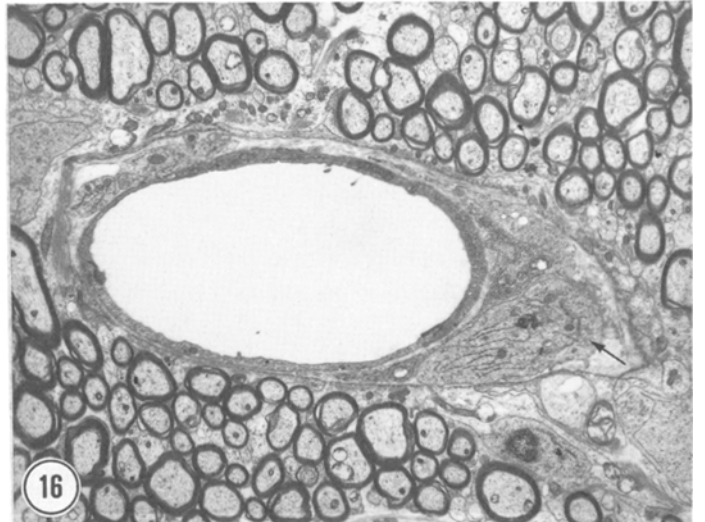
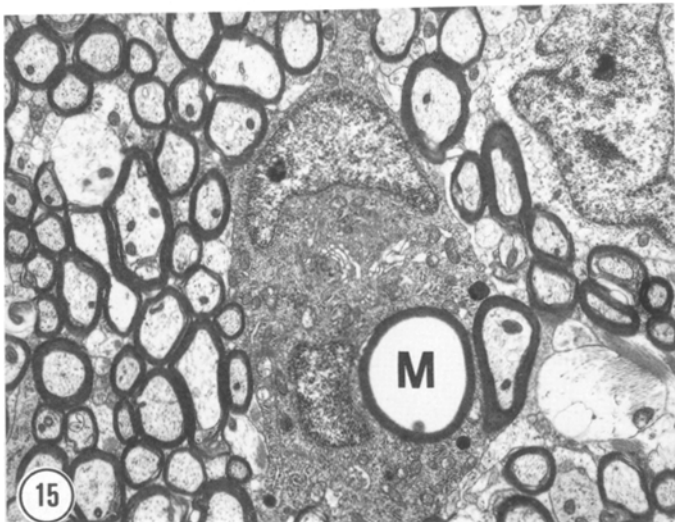
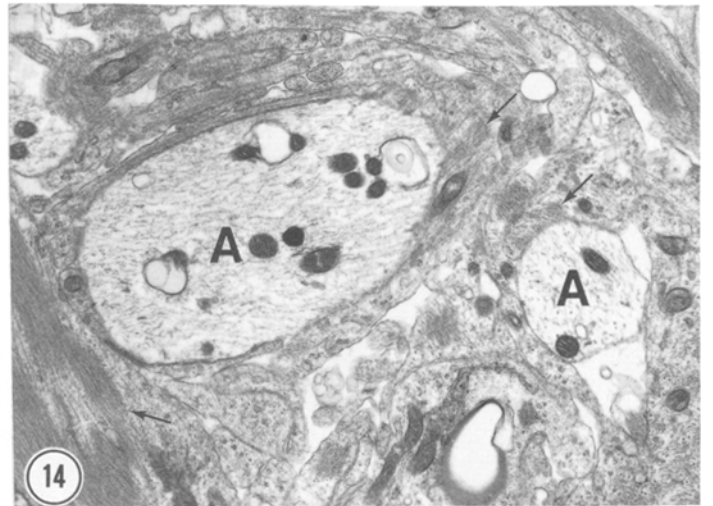
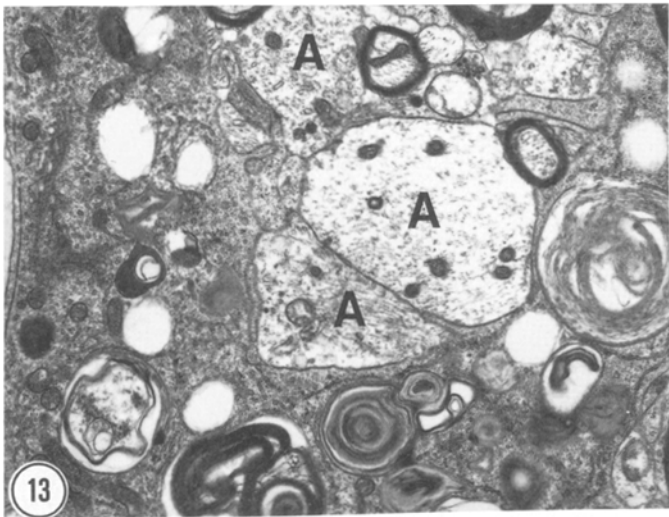
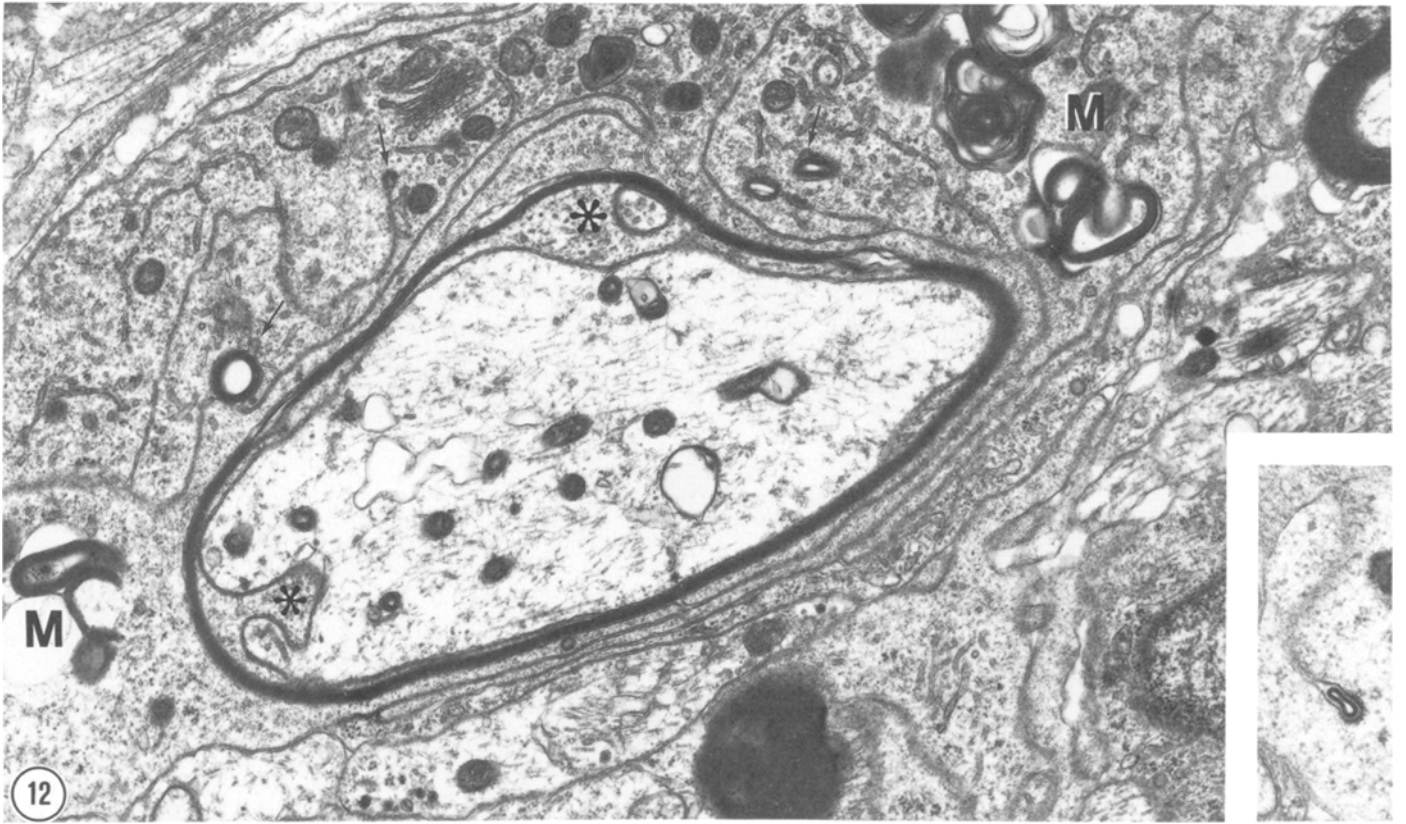
Fig. 7. A severely swollen, demyelinated spheroid sits among other intact myelinated fibres. $\times 9300$.

Fig. 8. At the surface of the fourth ventricle a macrophage and an ependymal cell contain canine distemper viral nucleocapsids (arrows). $\times 6300$.

Fig. 9. Reactive astrocyte (A) extends filament-bearing processes (arrows) among fascicles of myelinated axons. $\times 3100$.

Fig. 10. Phagocytic, hypertrophic astrocyte contains myelin debris (M). $\times 5100$.

Fig. 11. Nucleus of a microglial cell (M) with clumped heterochromatin; cytoplasmic profiles contain characteristic long profiles of rough endoplasmic reticulum (arrows). Notice one naked axon (A) and another (Ax) from which myelin is being stripped (arrowhead). $\times 6300$.



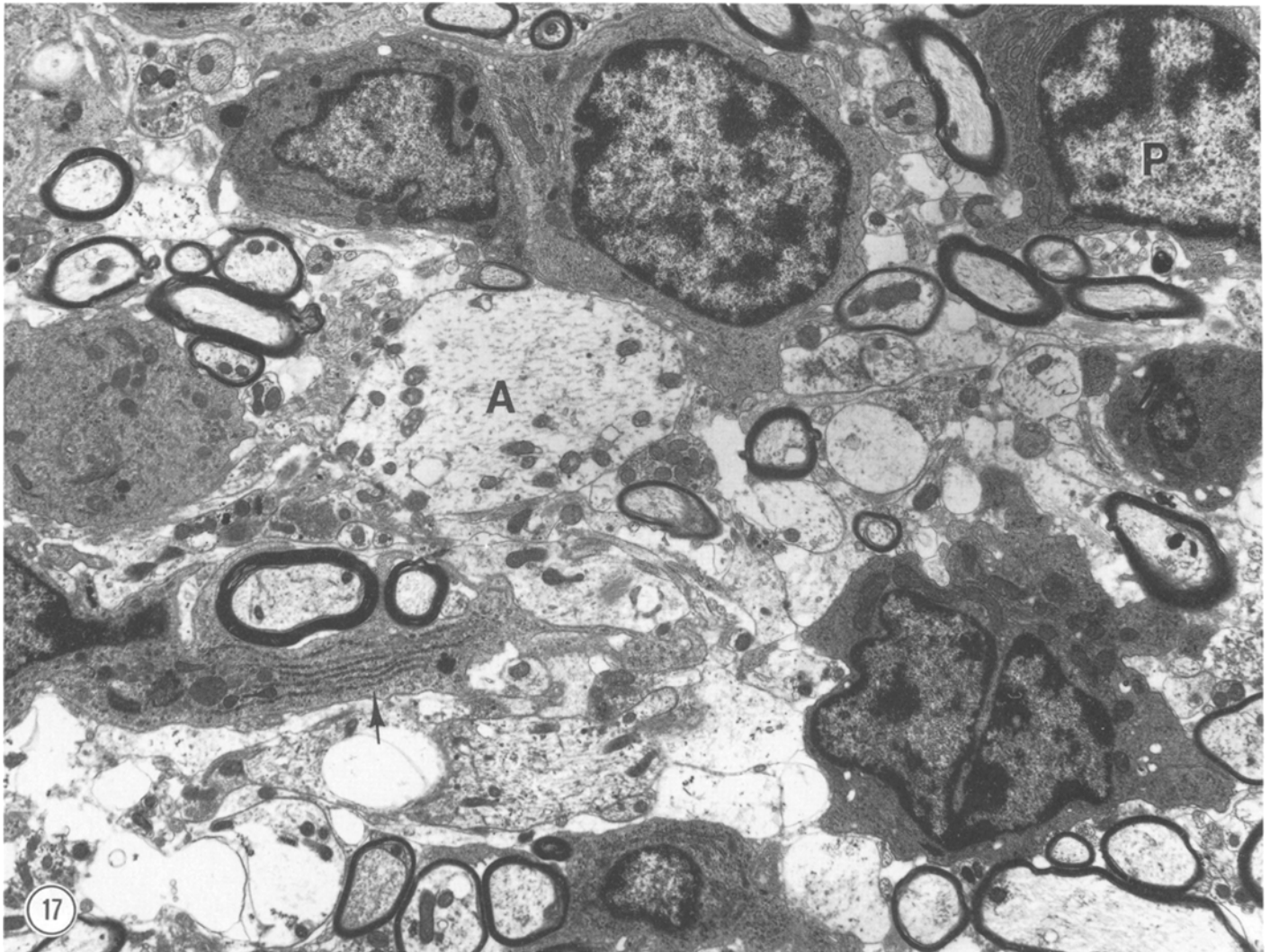


Fig. 17. A variety of mononuclear cells (plasma cell P) infiltrate a plaque. Notice the process of a macrophage with characteristic long profiles of rough endoplasmic reticulum (arrow). Although the plaque contains a demyelinated axon (A), many fibres are still ensheathed. Some with thin sheaths may be in the process of remyelination. $\times 7100$.

major dense line or intraperiod line. However, Higgins *et al.* (1982a) identified the intraperiod line as this site. This ballooning change may reflect intramyelinic oedema and is very similar to that seen in certain toxicities, such as triethyl tin, cuprizone and ethidium bromide, in which conditions the split

occurs at the intraperiod line (Aleu *et al.*, 1963; Ludwin, 1978; Yajima & Suzuki, 1979). In general the ballooned sheaths were empty, but occasionally contained a flocculent material, perhaps proteinaceous oedema fluid. Furthermore, it was quite common to find that the axon, about which the

Fig. 12. Active demyelination of an axon by cytoplasmic processes of a macrophage which contains abundant myelin debris (M). Note that macrophage processes have extended inside the sheath (asterisks) and have focally distorted the axon. Occasional coated pits are seen (arrows). $\times 18400$. Insert shows a myelin droplet within a coated pit. $\times 26000$.

Fig. 13. Three demyelinated axons (A) are enclosed by the processes of a typical macrophage (microglial cell) which contains myelin and myelin breakdown products. $\times 9300$.

Fig. 14. Two naked axons (A) are encircled by astrocyte processes. Bundles of intermediate filaments are numerous (arrows). $\times 9300$.

Fig. 15. A dark oligodendrocyte contains a large loop of myelin (M). Astrocyte cell body and processes to the right. $\times 5100$.

Fig. 16. In an area of intact white matter, profiles of a cell are seen in the perivascular compartment (arrow). $\times 3500$.

ballooning had occurred, was distorted; compression by oedema fluid within the ballooned sheath may be the explanation.

Spongiform white matter change has long been recognized from light microscopic studies of CDE (Campbell, 1957; Innes & Saunders, 1962), but as yet has not been put into the context of the developing lesion. Similar spongiform change has been described in association with other demyelinating diseases including Semliki Forest virus and mouse hepatitis virus infections of mice (Sheahan *et al.*, 1983; Lavi *et al.*, 1984), herpes simplex virus infection of rabbits (Kristensson & Wisniewski, 1978) and some forms of experimental allergic encephalomyelitis (EAE) in rats (Lassmann *et al.*, 1980). In some areas, myelin sheath ballooning was associated with more degenerative changes and axonal spheroid formation. Spheroids were also often partially or totally denuded. Higgins *et al.* (1982a) have referred to this process of concurrent axonal and myelin damage in CDE as 'complex necrosis'.

The early spongiform change is accompanied and perhaps preceded by a local proliferation of astroglial and microglial cells (Summers *et al.*, 1979; Van-develde *et al.*, 1982, 1983; Higgins *et al.*, 1982b). Although the ultrastructural features of the microglial cell are well documented (Mori & Leblond, 1969), its source and ultimate function are still strongly debated (Schelper & Adrian, 1986). The mesodermal derivation of microglia is now less secure (Kitamura *et al.*, 1984). Central to the issue is whether all CNS macrophages are of monocyte origin or whether these microglial cells are indeed a local source of phagocytes. From this study it appeared that cells compatible with reactive microglia, their processes often conveniently identified ultrastructurally by tubuloreticular inclusions within their endoplasmic reticulum, most commonly performed the process of myelin stripping and phagocytosis. This occurred in the absence of flagrant perivascular cuffs of mononuclear cells (such as monocytes). However, in evaluating blood vessels for such cells we sometimes noted swollen pericytic cells or extravascular, flattened macrophage-like cells in the perivascular compartment; pericytes may contribute to the pool of local macrophages (Baron & Gallego, 1972) and have been designated by others the 'pericytic microglia'.

A second glial cell which reacts early in these lesions is the astrocyte. We found that the hypertrophic (protoplasmic?) form of these cells revealed a phagocytic potential, an observation made previously in this disease by Raine (1976) and Higgins *et al.* (1982b) and in other circumstances, both *in vivo* (Ludwin, 1978) and *in vitro* (Kusaka *et al.*, 1985, 1986). Although astrocytes are conventionally thought of as glial cells involved in the repair process, we have previously suggested that their proliferation pre-

ceded myelin loss in CDE (Summers *et al.*, 1979). Indeed this observation is central to the argument that these cells are fundamentally involved in the process of demyelination in this canine disease. A proliferation of astroglial cells could follow direct virus infection of these cells. However, a diffuse astrocytosis also occurs early in non-infectious demyelinating diseases, such as EAE (Smith *et al.*, 1983), perhaps as a consequence of widespread tissue oedema.

The pathogenesis of demyelination in CDE is still unclear. Compelling evidence from ultrastructural studies of CDV-induced oligodendrocyte necrosis is lacking. Indeed electron microscopic demonstration of oligodendrocyte infection was not offered by Wisniewski *et al.* (1972), McCullough *et al.* (1974), Summers *et al.* (1979, 1984) or Higgins *et al.* (1982b). Raine (1976) described viral inclusions in 'occasional oligodendrocytes'. Occasional degenerating or necrotic cells were found in the white matter lesion in this study (noted also by Raine, 1976) and some or all may be oligodendrocytes. Thus, although rapid CDV-induced cytolysis must be considered, the absence of acute cytolysis of other infected neuroectodermal cells makes this proposition unappealing. It is further possible that oligodendrocytes are sites of defective viral infection with failure of viral assembly. Clarification of this latter possibility will require immunoelectron microscopic studies.

In contrast to oligodendrocytes, astroglial infection by CDV is readily and consistently demonstrated by all authors and perhaps is the primary target in white matter. It is recognized that astrocytes are important in maintaining ionic homeostasis within the neuroparenchyma (Duffy, 1983). Furthermore, they appear to contribute specifically to stability at the node of Ranvier (Raine, 1984a; French-Constant *et al.*, 1986; Waxman, 1986). Indeed Sims *et al.* (1985) suggested that the perinodal astrocyte process may play a role in the development and maintenance of central myelin. Accordingly, we propose that metabolic disturbances resulting from astrocyte infection may initiate the focal oedema and myelin ballooning that marks the initial white matter injury in CDE. Myelin ballooning could well have consequences for other, still compact internodes, maintained by the same oligodendrocyte. Subtle alterations, perhaps expressed at the surface of these internodes may attract local macrophages (microglia, astroglia) which invade and demyelinate these fibres. Lampert (1978) has commented that this myelin stripping is a non-specific pattern of demyelination that always follows damage to myelin sheaths or myelin-supporting cells. The concept of bystander demyelination, whereby the release of inflammatory mediators non-specifically injures myelin sheaths would seem to have little support from this study. We commonly observed fields of

mixed demyelinated and intact axons in immediate proximity, indicating that loss of the sheath involved a selective rather than haphazard process. Higgins *et al.* (1982b), in their studies of demyelinating CDE, came to the same conclusion.

Whereas microglial and astroglial cells constituted the most important source of macrophages, very rare cases of myelin phagocytosis by oligodendrocytes was encountered. This, apparently, is not a novel observation and has been recorded in lysolecithin-induced demyelination (Triarhou *et al.*, 1985) and following Wallerian degeneration in the CNS (Cook & Wisniewski, 1973). It is probable that blood monocytes constitute an important source of phagocytes during the inflammatory phase of demyelination.

The recognition of endocytosis of myelin droplets into clathrin-coated pits on the surface of macrophages was of interest. This process also has been recorded in other primary demyelinating diseases including MS (Raine, 1984b), EAE (Epstein *et al.*, 1983) and JHM virus encephalomyelitis (Fleury *et al.*, 1980). It is clear that these myelin diseases of animals and man have both unique and shared features.

The fate of demyelinated axons was of considerable interest. Evidence of remyelination was found, although not in great abundance, probably in concurrence with earlier studies (McCullough *et al.*, 1974; Higgins *et al.*, 1982b). This may relate to the apparent replacement of mature (medium and dark) oligodendrocytes by the light variety in well-established white matter lesions. Light oligodendrocytes have the most active mitotic activity (Mori & Leblond, 1970) and are probably progenitors of the dark, mature forms which contain the organelles (endoplasmic reticulum, Golgi) associated with synthetic functions. Furthermore, demyelinated axons in the loosened neuropil were invested by astroglial processes which sometimes formed spiral wrapping as others have noted in CDE (Wisniewski *et al.*, 1972; Higgins *et al.*, 1982b). This astroglial response may recapitulate development of the CNS wherein astroglial processes ensheath developing axons prior to their investment by cytoplasmic extensions of the oligodendroglia (Nagashima, 1979). This has also been shown in organotypic cultures of the CNS (Munoz-Garcia & Ludwin, 1986). Whether

in a reparative state this astroglial reaction ultimately aided or hindered remyelination is unknown, but our impressions would favour the latter interpretation. Tapered myelin sheaths and aberrantly located lateral loops which we encountered, albeit rarely, have been observed in lesions of MS (Prineas, 1975; Prineas & Connell, 1979), and in Balo's concentric sclerosis (Moore *et al.*, 1985), a probable variant of MS, and are taken to indicate myelin repair.

The role of the lymphoplasmacytic infiltrate which appears in the more chronic phase of CDE has not been defined. In general, evidence that immune mechanisms play a primary role in myelin injury in CDE are lacking (Cerruti-Sola *et al.*, 1983). In marked contrast, white matter lesions in CDE are directly associated with the presence of the virus, or its proteins, in the tissue (Vandeveldel *et al.*, 1985; Mitchell, 1987). Virus was not found in non-lesion areas (Wisniewski *et al.*, 1972). We concur with the interpretation offered by Bollo *et al.* (1986) that these infiltrating cells are responding to viral rather than myelin antigens, and are attempting to clear the brain of the persistent infection. It appears that if the immune response is prompt, clearance can be effected (Appel *et al.*, 1982) and the animal recovers. If the inflammatory reaction is delayed, the lesions expand and may change from demyelinating to necrotizing (Wisniewski *et al.*, 1972). The capacity of astrocytes to present antigens to infiltrating T-cells may trigger this destructive process (Sun & Wekerle, 1986), resulting in the release of lymphotoxins which produce tissue necrosis. Clarification of these events and related questions (are the astrocytes Ia positive?) should prove of interest to investigators of demyelinating diseases of the CNS.

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