Acta Neuropathol (1991) 82: 60 – 65

Acta Neuropathologica
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P_2-peptide induced experimental allergic neuritis: a model to study axonal degeneration*

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Received November 13, 1990/Revised, accepted March 18, 1991

Summary. In experimental allergic neuritis (EAN) severity of clinical disease and pathology correlate with the dose of antigen (Hahn et al., Lab Invest 59:115–125, 1988). To avoid axonal membrane contamination of the antigen, EAN was induced with a synthetic peptide, corresponding to residues 53–78 of bovine P_2 myelin protein. Severity of EAN correlated with the dose of peptide in the inoculate. The relationship between demyelination, inflammation and axonal degeneration was studied. Low doses resulted in pure demyelination. Axonal degeneration occurred only with high doses of antigen and in association with very active mononuclear inflammation. The role of macrophages in producing axonal damage is discussed.

Key words: Experimental allergic neuritis: P_2 myelin peptide – Demyelination – Axonal degeneration

Experimental allergic neuritis (EAN), an immune-mediated inflammatory demyelinating disease of the peripheral nervous system [34] can be induced in Lewis rats by sensitization with homogenated nerve, purified myelin, and bovine myelin P_2 protein [17, 25, 27]. The neuritogenic determinant of bovine P_2 myelin protein has been located within the residues 53–78 [33] and recent observations suggest that only residues 62–65 may be necessary for EAN induction [31]. Olee and colleagues [22] have demonstrated that residues 61–70 of P_2 protein represent the minimum peptide-length requirement for the T cell epitope for EAN. Addition of a further single amino acid, the equivalent of residues 60–70 of P_2 protein, significantly enhanced the clinical severity and pathology of induced EAN. A comparably severe form of EAN has been induced in young Lewis rats by sensitization with a synthetic peptide SP_{26} corresponding to the amino acids 53–78 of bovine P_2 protein [26]. Roots and sciatic nerves showed typical inflammatory-mediated demyelination.

Current research in EAN has elucidated the importance of T lymphocytes and macrophages in effecting the immune-mediated attack on the myelin sheaths (reviewed in [11]). Accompanying damage to nerve axons has long been recognized [18, 19, 21, 30] yet its etiology remains unknown. Axonal degeneration also occurs in the human demyelinating neuropathy, the Guillain Barré syndrome, and contributes significantly to the residual permanent disability [1, 5, 24]. In EAN induced with whole myelin extracts, the severity of disease and the degree of axonal degeneration increased with higher doses of antigen [9]. We concluded that the axonal degeneration was possibly caused by mediators of the severe accompanying inflammatory response. However, a specific immune attack on axons evoked by axonal membrane contamination of the myelin extract could not be excluded. To eliminate this possibility, we induced EAN with varying doses of a small synthetic peptide SP_{26} which corresponds to the amino acid sequence 53–78 of bovine P_2 myelin protein. We found an excellent correlation between severity of clinical disease and pathological changes with the dose of antigen. Low doses produced a graded pure inflammatory demyelination. However, with higher doses of antigen there was severe edema, inflammation and abundant axonal degeneration.

Material and methods

The synthetic peptide of bovine P_2 myelin protein (SP_{26}) was purchased from the Tripartite Microsequencing Centre, University of Victoria, Victoria, BC.

EAN was induced in young male Lewis rats (200–250 g) using a synthetic peptide (SP_{26}), which corresponds to the amino acid sequence 53–78 of bovine P_2 myelin protein. The inoculate, composed of 25, 50, 75 and 100 μg of peptide, respectively, in saline plus complete Freund's adjuvant (CFA), was injected intradermally

* Supported by a grant of the Muscular Dystrophy Association of Canada and the Medical Research Council of Canada

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into the left hind footpad (four rats/group). Four control rats were injected with saline and adjuvant only. The animals were weighed daily and scored for signs of clinical disease: 0, normal; 1 limp tail; 2, abnormal gait; 3, mild paraparesis; 4, severe paraparesis; 5, paraplegia; 6, paraplegia with forelimb involvement; 7, paraplegia with forelimb involvement and respiratory distress; 8, moribund or dead. On days 20–21 post immunization (p.i.) three animals in each group were anesthetized with pentobarbital and killed by intracardiac perfusion with 150 ml phosphate-buffered saline containing 1.5 ml heparin, followed by 150 to 200 ml 2.5% glutaraldehyde in 0.1 M phosphate buffer. Samples were taken from the lumbar spinal cord, lumbar roots and dorsal root ganglia, the cauda equina and both sciatic nerves at proximal and distal levels. Tissues were immersed in the same fixative for another 3 h, osmicated, dehydrated in graded alcohols and propylene oxide and embedded in Epon/Araldite resin. One animal in each group was observed clinically until days 25–27 p.i. while recovering from the acute phase of EAN.

The clinical course and the pathological changes of peptide-induce EAN (50 μg SP_{26} in saline plus CFA) were characterized at days 10, 12, 14, 16, 18 and 24 p.i. (two rats/group). They were killed and sampled in the same manner as detailed above. Representative toluidine blue-stained sections from each sample were examined under the light microscope. The degree of demyelination, inflammation, edema and axonal degeneration was graded blind on the following scale: *demyelination:* 1+, a few demyelinated axons perivenular or scattered; 2+, many foci of perivenular demyelination; 3+, extensive demyelination perivenular and confluent; *inflammation:* 1+, a few scattered mononuclear inflammatory cells often subperineurial; 2+, perivenular cuffing with mononuclear inflammatory cells; 3+, extensive multifocal perivenular cuffing and wide spread endoneurial inflammation; *edema:* 1+, subperineurial edema; 2+, moderate generalized endoneurial edema; 3+, severe endoneurial edema; *axonal degeneration:* 1+, a few fibres undergoing axonal degeneration; 2+, numerous fibres undergoing axonal degeneration; 3+ extensive axonal degeneration throughout the nerve fascicle. An average of 200 perivenular areas per animal group and site (lumbosacral roots and sciatic nerves) were scored and the mean pathological grades for the respective parameters calculated. Ultrathin sections were obtained from selected samples. These were double stained with uranyl acetate and lead citrate and viewed with an electron microscope (Philips 410).

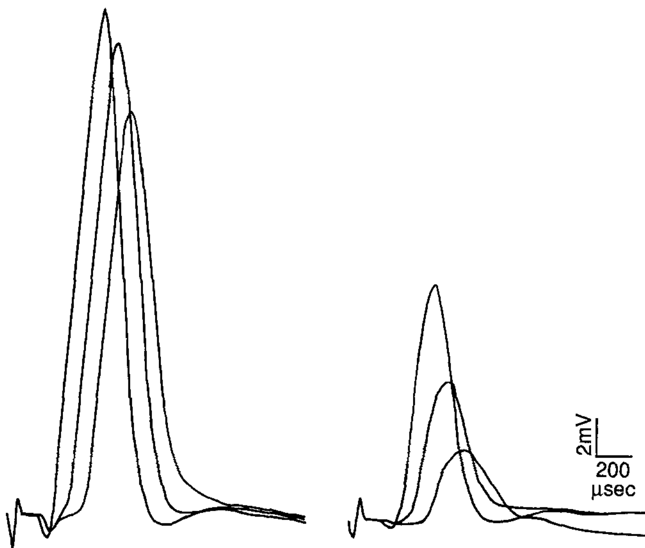


Fig. 1. Monophasic recordings at 10-, 15- and 20-mm distance in normal *(left)* and experimental allergic neuritis (EAN; *right*) rat dorsal roots. The reduction in amplitudes of the compound action potentials with distance in EAN root recordings indicates conduction block

Physiological studies

Recordings were performed on days 14, 15 and 16, respectively, following immunization with 50 μg SP_{26} (three rats with clinical grade ranging between 1 and 3). Rats were anesthetized with Nembutal, a lumbar laminectomy was performed and the rat was suspended in a recording frame. The dura was opened and the roots suspended in warmed (34 °C) oxygenated silicone oil. Suitable dorsal roots were suspended individually on recording, ground and stimulating electrodes. The proximal recording electrode was moveable. The root was crushed just proximal to the distal recording electrode to allow monophasic recording and the root was cut proximal and distal to the electrode sites just prior to recording. The proximal electrode was moved so that there were three recording distances: 10, 15 and 20 mm. Each dorsal root was stimulated supramaximally and eight responses averaged at each distance. Control recordings were made from dorsal roots of two normal rats of comparable age and weight.

Results

Characterization of P_2-peptide induced EAN

Clinical observations

A medium dose of antigen (SP_{26} 50 μg) was used to delineate the clinical course and the development of pathological changes in P_2-peptide induced EAN. Animals began losing weight around day 11 p.i. 2 days prior to overt clinical signs. Ruffled fur, tail weakness and gait ataxia were observed on days 12 and 13 p.i. The hind limb weakness progressed during the following 4 days, to reach a maximum around days 16–17 p.i. Maximal impairment in individual animals ranged from moderate gait abnormality to paraplegia. However, even severely affected animals improved considerably and remained with only mild residual gait disturbance.

Physiological observations

Recordings were made from 13 dorsal roots of EAN rats and 5 dorsal roots of control rats. The mean conduction velocity in the EAN roots was 44.4 m/s (± 1.3) and in the control roots 45.4 m/s (± 2.4). These were not significantly different. The amplitudes of the compound monophasic action potentials were measured at each recording position. In the EAN roots, the action potential at 15 mm was 79.2 (± 2.6)% of that at 10 mm and at 20 mm it had dropped to 56.3 (± 4.1)%. In the control roots, the action potentials at 15 mm had dropped to 88.4 (± 3.9)% and by 20 mm it had dropped to 71.4 (± 6.3)%. Figure 1 illustrates a representative recording. These values differ between the control and EAN roots at a level of $P < 0.05$.

These results indicate that the conduction velocity is not significantly different between the EAN and normal roots in these mildly affected EAN rats at this early stage of the disease. However, there is a significant drop in the

amplitude of the compound monophasic action potential with distance, indicating conduction block.

Pathological observations

Tissues sampled on day 10 p.i. appeared normal. However, on day 12 p.i. single nerve roots of the cauda equina showed diffuse edema, scattered mononuclear cell infiltrates and early invasion of Schwann tubes by macrophages with signs of active myelin stripping. Lesions in sciatic nerves were less advanced, with primarily diffuse edema and scattered mononuclear cells. On day 14 p.i. the leptomeninges were infiltrated by lymphocytes. There was heavy multifocal perivenular cuffing of mononuclear cells in many nerve roots, with associated severe proteinaceous edema. Many Schwann tubes were invaded by macrophages, which were actively stripping and degrading myelin. Some myelin sheaths showed vesicular disruption or were markedly ballooned. In sciatic nerves, infiltrating mononuclear cells were more numerous and they were making contact with myelinated axons. On day 16 p.i. many foci of macrophage-associated perivenular demyelination were now well established, both in ventral and dorsal roots. Axons were totally demyelinated and were surrounded by healthy-appearing Schwann cells. Lipid-laden macrophages appeared to be leaving the Schwann tubes and were aggregating around small venules. Disease was also well established in sciatic nerves, which showed prominent multifocal lymphocytic perivenular cuffing and diffuse endoneurial infiltrates of mononuclear cells, mainly of activated macrophages. There were many demyelinated axons, usually in the vicinity of endoneurial venules, but also scattered among the heavy inflammatory infiltrates. On day 18 p.i. the disease process in nerve roots was beginning to subside. There were only few infiltrating lymphocytes and macrophages laden with small lipid droplets were gathering around endoneurial vessels. Many axons in the vicinity of small venules were totally demyelinated and were surrounded by Schwann cells, whose cytoplasm contained ample rough endoplasmic reticulum and prominent Golgi complexes. Others showed early signs of remyelination with a few layers of compacted myelin lamellae. In sciatic nerves, there was still actively ongoing widespread inflammatory demyelination and a few scattered fibres were undergoing acute axonal degeneration. On day 24 p.i. the disease in nerve roots had become quiescent. Lymphocytes and macrophages had largely disappeared. Many foci of thinly remyelinated axons, in perivenular distribution, were indicative of extensive prior disease and of actively ongoing repair. The same was true for the sciatic nerves. The endoneurium was still distended by edema and macrophages, heavily laden with myelin debris, were scattered among the nerve fibres and were gathered around endoneurial vessels. Many thinly myelinated axons were indicators of the preceding widespread demyelination. There was little evidence of nerve fibre loss and the repair process appeared to be ongoing.

Dose response in P_2-peptide induced EAN

Clinical observations

All rats immunized with varied doses of SP_{26} developed signs of EAN which were similar in course and severity to disease produced by sensitization with nerve homogenate [8], with myelin [9] or with P_2 myelin protein (Hahn, unpublished results). As a first indicator of disease, animals began to lose weight around day 10 p.i. The weight loss continued throughout the period of maximal illness and paralleled the severity of disease. The mean clinical scores for each group of animals are illustrated in Fig. 2. The severity of clinical disease correlated positively with the dose of peptide in the inoculate. Animals given 50–100 μg SP_{26} showed early signs of disease, ruffled fur and a limp tail, beginning on days 12 and 11 p.i., respectively. This was followed by progressive hind limb weakness, which reached a maximum between day 16 to day 19 p.i. with subsequent gradual improvement. Disease was milder and slightly delayed in animals injected with 25 μg peptide. At the end of the observation, days 25–27, animals inoculated with 75 and 100 μg remained with a moderate paraparesis, whereas animals inoculated with a lower dose had totally recovered.

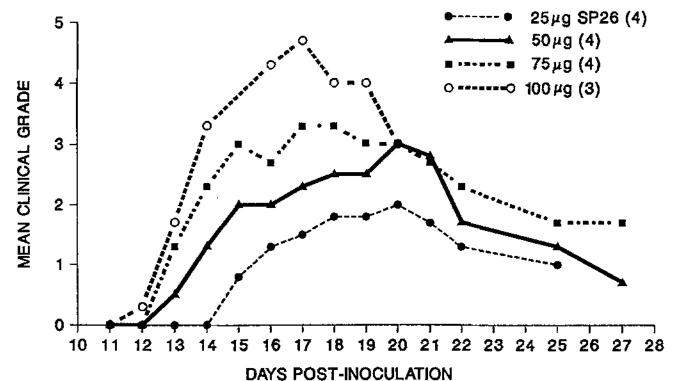
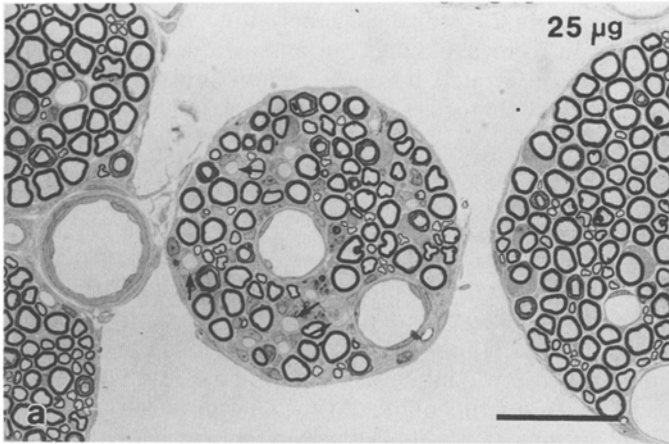


Fig. 2. The severity of clinical EAN in Lewis rats correlates positively with the dose of peptide used for immunization

Pathological observations

Examination of dorsal root ganglia, lumbosacral nerve roots and sciatic nerves showed the typical lesions of inflammatory perivenular demyelination, characteristic for EAN in all animals. Changes were restricted to the

Fig. 3. Light micrographic illustrations of lumbosacral nerve roots (*a, c, e, g*) and of sciatic nerves (*b, d, f, h*). Perivenular macrophage-associated demyelination (*arrows*) increases in severity with higher doses of peptide. Accompanying axonal degeneration (*arrowheads*) is seen only with high doses of peptide. It is particularly severe in areas of dense inflammation in sciatic nerves (*f, h*). Toluidine blue-stained sections; bar = 50 μm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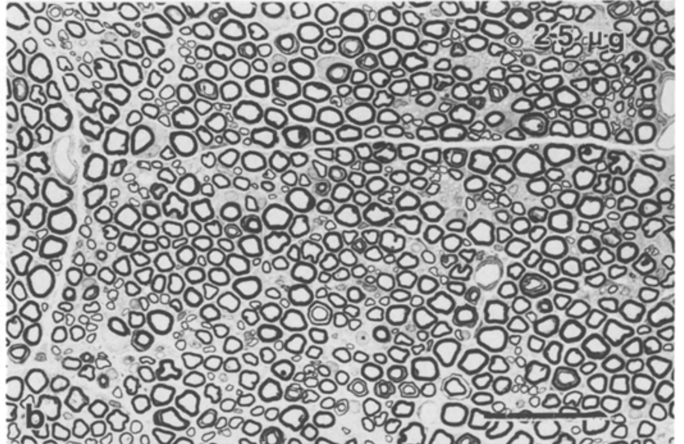
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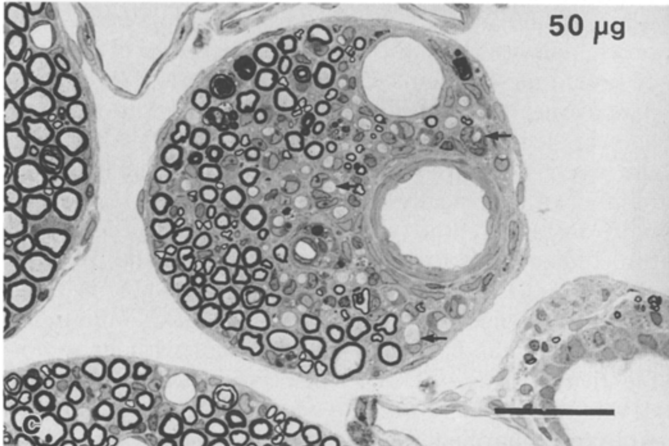
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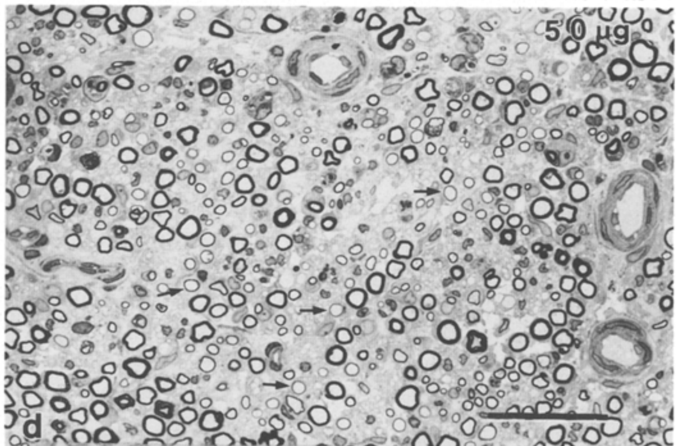
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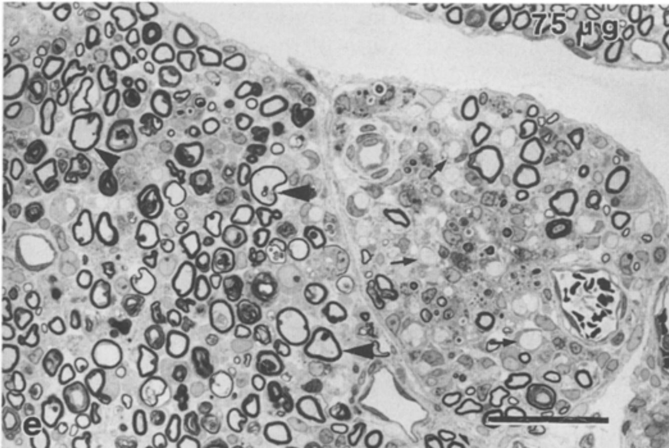
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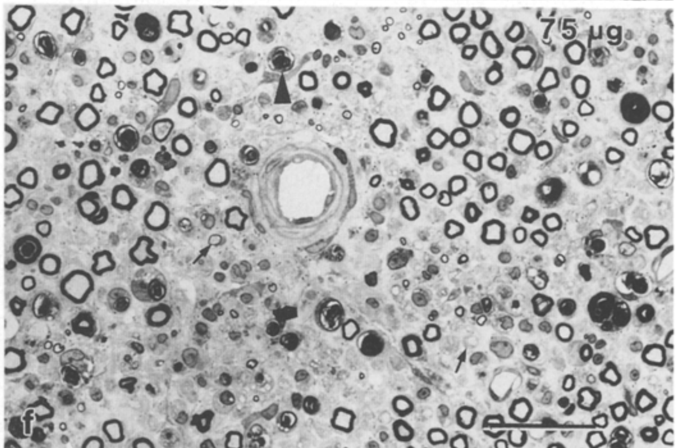
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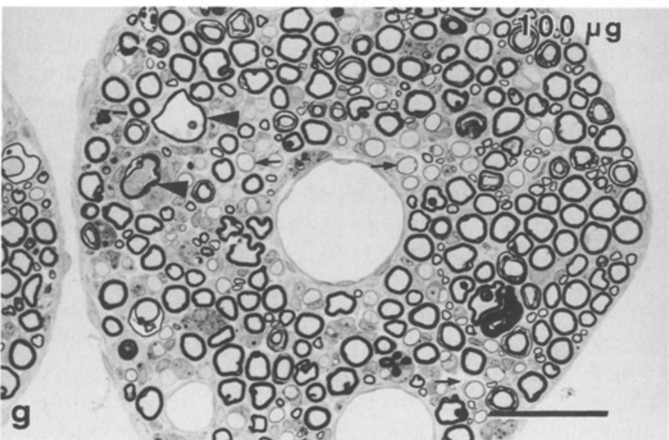
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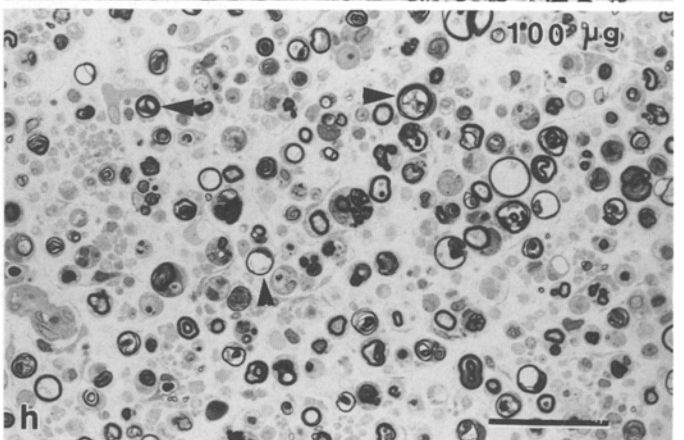
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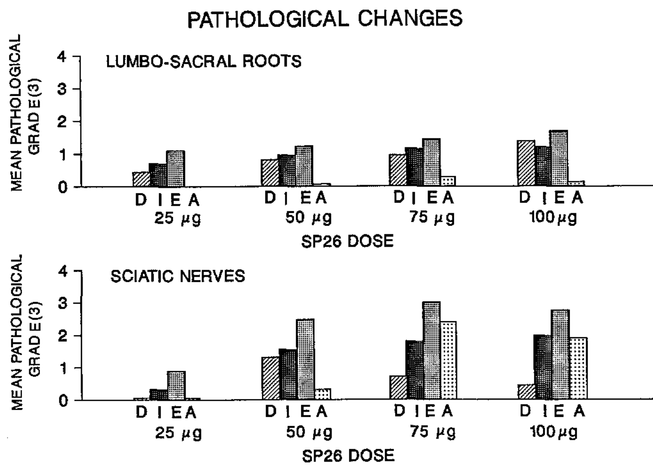

Fig. 4. Illustrations of the mean grade of demyelination *(D)*, inflammation *(I)*, edema *(E)* and axonal degeneration *(A)* in roots and sciatic nerves of Lewis rats immunized with varying doses of peptide SP_{26}. Low doses of antigen induce almost pure demyelination. Inflammation, edema and axonal degeneration are severe with higher doses

peripheral nervous system, the spinal cords being normal, with the exception of the occasional leptomeningeal lymphocytic infiltrates. Representative illustrations of the findings in nerve roots and sciatic nerves are given in Fig. 3. The histopathological findings are detailed by group below. The mean pathological scores for each group are graphically shown in Fig. 4.

25 µg peptide. The lumbosacral nerve roots showed scattered foci of perivenular demyelination in association with edema, mild lymphocytic infiltrates and macrophages containing myelin debris. There was no axonal degeneration (Fig. 3a). Ventral roots appeared more prominently affected. Sciatic nerves were either normal or showed only mild endoneurial edema, scarce perivenular and subperineurial mononuclear inflammatory cells and a few scattered demyelinated axons (Fig. 3b).

50 µg peptide. Typical multifocal perivenular demyelination was much more widespread in ventral and dorsal lumbosacral roots (Fig. 3c). Similarly, sciatic nerves showed much more prominent perivascular and diffuse endoneurial edema, mononuclear inflammatory cell infiltrates and associated demyelination and remyelination (Fig. 3d). There was little axonal degeneration.

75 µg peptide. Lumbosacral roots showed very active disease, with multifocal, perivenular and confluent macrophage-associated demyelination. In addition, in areas of prominent inflammation, there was also active nerve fibre degeneration (Fig. 3e). Sciatic nerves appeared distended by prominent edema and diffuse endoneurial inflammation, with scattered demyelinated axons. Acute axonal degeneration was prominent in areas of perivascular inflammation (Fig. 3f).

100 µg peptide. The pathological changes in nerve roots were similar to those of the previous group, showing extensive multifocal demyelination and variable, but relatively mild axonal degeneration. Sciatic nerves, however, were expanded by massive edema and were diffusely infiltrated by dense inflammation. In all but one animal there was almost total axonal destruction (Fig. 3h).

Discussion

We conclude from the observations of this study, that the severity of clinical EAN, even when induced with a very specific neuritogen, such as the synthetic peptide of P_2 myelin protein, may vary according to the dose of antigen in the inoculate. Low doses produced a milder and an almost purely demyelinative form of disease, whereas high doses provoked rapidly progressive and severe paralysis with the additional histological findings of severe nerve edema, florid inflammation and abundant axonal degeneration. Analogous observations have been made in the adoptive transfer model of EAN where the nerve pathology clearly varied with the injected number of specifically sensitized T cells [10, 15, 16]. In both instances, the specific immune responses are directed towards the myelin sheaths with the primary effector cells being activated macrophages [16, 19, 23]. The prominence of macrophages in the EAN inflammatory lesion has been clearly identified with immunoreactive markers [7, 29]. Their biological role in the development of EAN and in demyelination was demonstrated by pharmacological suppression of their secretory products [12, 13]. The disease became clearly attenuated, whereas activation of macrophages with interferon-gamma had the opposite effect [14]. Phagocytic macrophages are, thus, identified as the primary cells engaged in the stripping and digestion of myelin and are possibly targeted to the myelin sheath by the prior deposition of complement components $C_{5b\text{-}9}$ (Stoll, personal communication). The importance of complement activation in EAN has been previously demonstrated [6]. This may lead to an opsonization of myelin with subsequent phagocytosis by macrophages during segmental demyelination in a manner similar to that described during Wallerian degeneration [2–4, 7, 28, 32].

Macrophages also appear to be the major effectors of axonal destruction in Wallerian degeneration in rodents [2, 3, 28]. Lunn and colleagues [20] found that in the C57BL/6/OLa mouse, axons in the distal stump of transected sciatic nerves appeared intact and were able to conduct electrical impulses for at least 14 days. They postulated that this might be due to the observed lack of recruitment of macrophages into the nerve stump. Treatment of normal mice with a monoclonal antibody against the complement type 3 receptor, which prevents macrophage recruitment, also resulted in prolonged survival of transected axons. Macrophages, thus, appear to take part in axonal degeneration. The mechanisms by which recruited cells initiate axonal destruction remains to be established. In EAN, axonal degeneration is always observed in association with severe endoneurial

inflammation and it seems very plausible that axons might be damaged by secretory products of activated macrophages.

Acknowledgements. The authors wish to thank Mrs. J. Morehouse for the preparation of the manuscript.

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