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Studies of activated microglia and macrophages in lumbosacral spinal cord following an intraperitoneal injection of 6-aminonicotinamide into adult rats

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Summary. The anterior horns of the lumbosacral segment of the spinal cord in rats showed an extensive lesion following an intraperitoneal administration of 6-amino-nicotinamide (6-AN). Neuronal chromatolysis was observed in some of the large efferent neurons 1-7 days after the injection of 6-AN. The capability in their uptake of fluorogold and its retrograde transport was comparable to those in the normal rats. Small neurons presumably internuncial cells underwent degeneration. 8 weeks after 6-AN injection, all the surviving neurons appeared normal. 1 day after 6-AN injection microglial cells appeared activated as evidenced by the hypertrophy and expansion of their processes. A large number of macrophages were observed in the lesioned site 7 days after the administration of 6-AN. The activated microglia and macrophages showed intense immunoreactivity with the monoclonal antibody OX-42. The immunoreactivity declined with time so that by 4 weeks after the injection of 6-AN very weak immunoreactivity was seen on some very branched cells. A similar pattern of immunoreactivity was observed with the monoclonal antibodies OX-18 and OX-6. It was concluded from this study that neuronal chromatolysis and neuronal degeneration induced the expression of CR3 receptors (marked by OX-42) and MHC encoded antigens (marked by OX-18 and OX-6) in activated microglia and macrophages. With time the immunoreactivity decreased so that by 4 weeks after the administration of 6-AN only faint immunoreactivity was observed on some branched cells. Concurrent with a possible down regulation of surface antigens was the restoration of the motor activity of the extremities of the animals which were severely paralyzed 4-7 days after 6-AN injection.

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Key words: 6-Aminonicotinamide, Rat, Spinal cord, Antibodies, Macrophages

Introduction

6-amino-nicotinamide (6-AN) is an antagonist of nicotinamide (Johnson and McColl, 1955). A number of biochemical studies on the anti-niacin effect of 6-AN have been described (Dietrich et al., 1958). Morphologic studies pertaining to 6-AN neurotoxocity have also been reported in adult rodents (Sternberg and Philips, 1958; Horita et al., 1978). When administered in experimental animals, the antimetabolite is known to produce severe damage in the central nervous system (Schotland et al., 1965). A drastic neurological disturbance in rodents is the paralysis of their extremities (Sternberg and Philips, 1958; Horita et al., 1978). Associated with this motor deficit were degenerative changes in the anterior horns of the spinal cord (Horita et al., 1978). Horita et al. (1978) found neuronal chromatolysis in lesions in the cervical cord after an intraperitoneal injection of 6-AN in rats. Infiltration of macrophages at the site of lesion was also observed by the above mentioned authors. Furthermore, macroglial alteration presumably due to the toxic effects of 6-AN has also been noted (Chui and Garcia, 1979).

The present study was conducted to confirm neuronal degeneration in the anterior horns of the spinal cord in the lumbosacral segments and to verify the type of neurons affected after an intraperitoneal injection of 6-AN. The functional integrity of the anterior horn neurons was assessed by their capability to retrogradely transport a fluorescent dye. Another aim of this study was to examine the activation including immunophenotypic features of microglial cells and infiltrated macrophages associated with degenerative changes after the administration of 6-AN. For this purpose monoclonal antibodies which are known to label

macrophages and activated microglial cells in other parts of the central nervous system (Ling et al., 1990, 1991, 1992; Kaur and Ling, 1992) were used.

Materials and methods

Intraperitoneal injections

40 adult Wistar rats (about 200 gm) were used for the present study. Each rat was given a single intraperitoneal injection of 6-AN (Sigma Co. catalogue no. A0603) (10 mg/kg body weight in concentration of 5 mg/ml of physiological saline). 3 days after the injection of 6-AN, fluorogold (FG) (Fluorochrome Inc) was injected intraperitoneally (500 µl of 1% FG per rat). The rats were allowed to survive for 6 hours, 1, 4, 7, 10 days, 2, 3, 4 and 8 weeks after the injection of 6-AN.

Routine transmission electron microscopy

Following chloral hydrate anaesthesia, 4 rats which survived 1 day, 7 days and 8 weeks after the injection of 6-AN were perfused with a mixed aldehyde solution composed of 2% paraformaldehyde and 3% glutaraldehyde in 0.1M cacodylate buffer. Lumbosacral spinal cord and tibialis anterior muscle (to verify if there were any changes at the neuromuscular junctions) were removed. 50 µm vibratome sections of the spinal cord and the muscle were prepared and postfixed for 1 hour in 1% osmium tetroxide. Following dehydration in alcohol, the tissue blocks were embedded in Araldite mixture. For general survey, 0.5 µm thick sections were cut and stained with methylene blue. Ultrathin sections stained in lead citrate were viewed in a Jeol 1200EX electron microscope. For comparison, two normal rats were perfused and their spinal cords were processed in a similar manner.

Immunohistochemistry

The rats which survived from 6 hours to 8 weeks after an intraperitoneal (IP) injection of 6-AN, were anaesthetized with 7% chloral hydrate and were perfused with Ringer's solution until the liver and lungs were clear of blood. This was followed by perfusion with an aldehyde fixative composed of a mixture of periodate-lysine-paraformaldehyde according to the method of McLean and Nakane (1974) with a concentration of 4% paraformaldehyde. The perfusion lasted for 15 minutes after which the lumbosacral spinal cord was removed and kept in a similar fixative for 2 hours. Frozen transverse sections of the spinal cord of 40 µm thickness were cut and rinsed in phosphate-buffered saline (PBS). The sections were then incubated at room temperature (22 °C) with monoclonal antibodies OX-42 (Sera Lab, MAS 370), OX-18 (Sera Lab, MAS 101b) and OX-6 (Sera Lab MAS 043b) diluted 1:100 with PBS for 18 hrs. Vectastain ABC-kit (PK-4002, Vector Laboratories) against mouse IgG was used for subsequent detection of antibodies with 3,3'-diaminobenzidine as a substrate.

Some of the frozen sections of the spinal cord from each of the above rats were mounted on gelatinized slides, air dried and coverslipped with the non-fluorescent medium Entellan. The sections were examined and photographed in a Leitz Aristoplan photomicroscope equipped with a mercury lamp for fluorescence microscopy, using a wide band ultraviolet excitation filter (BP 355-425). The spinal cords of 4 normal rats (which received intraperitoneal injection of FG only) were processed in a similar manner as above for fluorescence microscopy and immunohisto-chemistry.

Immunoelectron microscopy

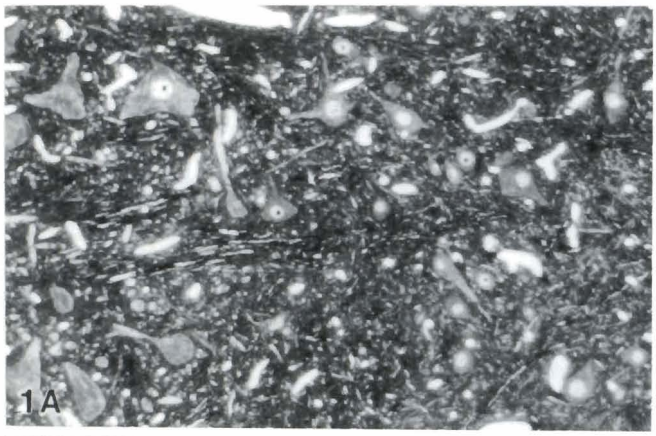
4 experimental rats which survived 7 days after an IP injection of 6-AN were used. Only animals of this post-injected interval were examined because they appeared most severely affected symptomatically. They were anaesthetized with 7% chloral hydrate and were perfused with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1M phosphate buffer for 15 minutes followed by a second fixative composed of periodate-lysine-paraformaldehyde (McLean and Nakane, 1974) for 15 minutes. Lumbosacral spinal cord was removed and kept in 0.1M phosphate buffer at 4 °C. 50 µm vibratome sections of the spinal cord were prepared and were incubated with monoclonal antibodies OX-42, OX-18 and OX-6 for immunocytochemical reaction in a similar manner as for light microscopy. The sections were post-osmicated in 1% osmium tetroxide in 0.1M phosphate buffer. The sections were dehydrated in a graded series of alcohol and embedded in Araldite mixture. Ultrathin sections were cut and stained with lead citrate and were examined in a Jeol 1200EX electron microscope.

Results

1 day after an IP injection of 6-AN, the rats became hypoactive and developed weakness at their extremities notably in their hind limbs. 4-7 days after the injection both of their hindlimbs were clearly paralysed. Beginning 2 weeks after 6-AN injection, their physical activity improved gradually so that between 4-8 weeks after the injection their motor activity was completely recovered.

Light microscopy

In the light microscope, the lumbosacral spinal cord in 6-AN injected rats showed dramatic degenerative changes in the anterior horns when compared with the normal animals. In the normal spinal cord the texture of the neuropil in the anterior horns was compact containing neurons of different sizes and various types of glial elements (Figs. 1A,B). 1 day after an IP injection of 6-AN, the gray matter was relatively compact. There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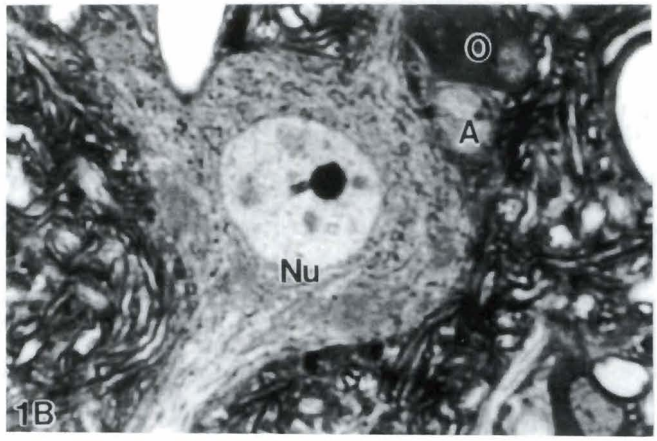

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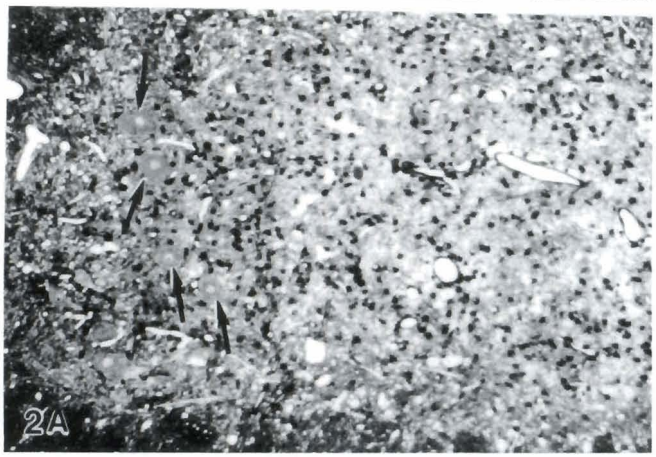
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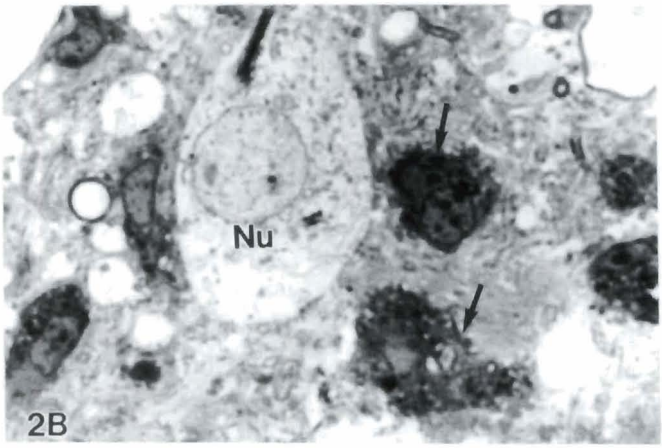

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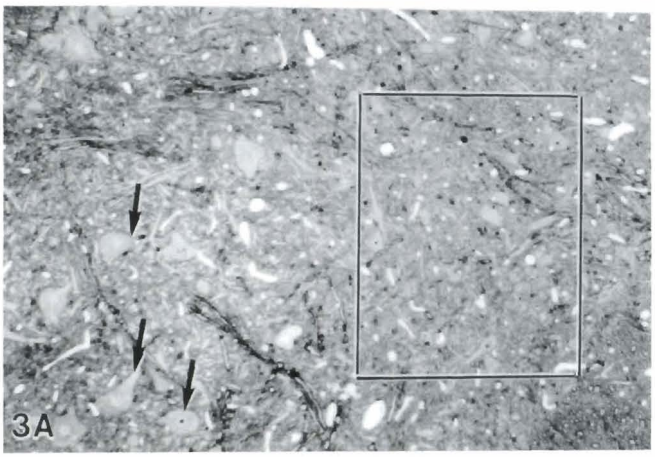
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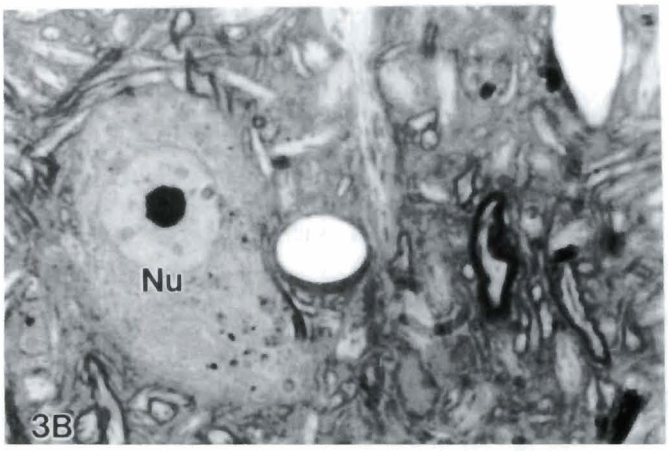

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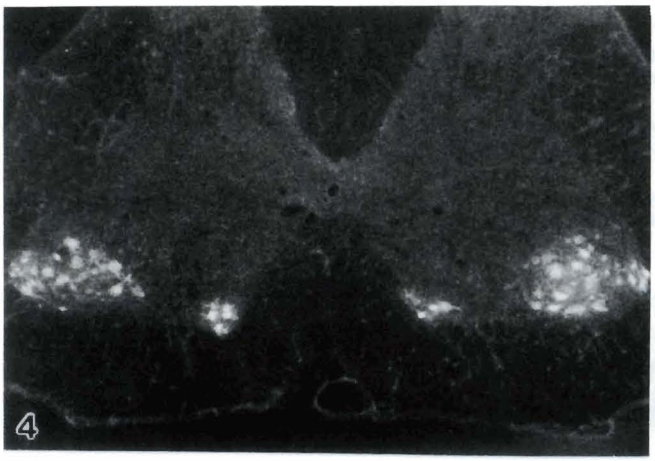
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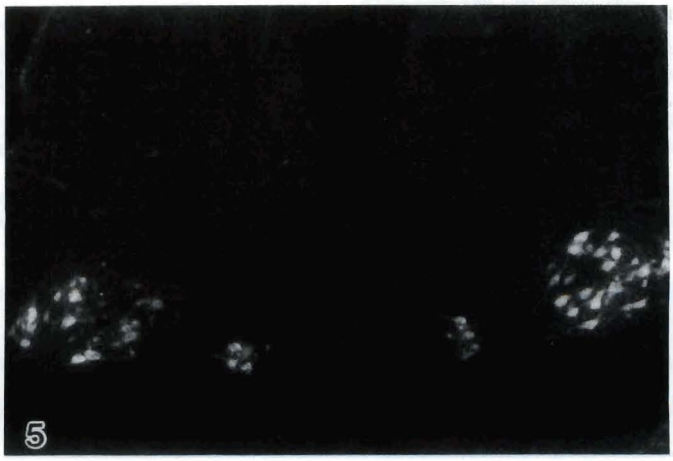
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Fig. 1. A. A portion of the anterior horn of the lumbosacral spinal cord of a normal rat. In the compact neuropil neurons of various sizes can be seen. x 140. **B.** An enlarged view of a small area of Fig. 1A showing a large neuron (Nu), oligodendrocytes (O), and an astrocyte (A). x 1,360

Fig. 2. A. Anterior horn of the lumbosacral spinal cord 7 days after an IP injection of 6-AN. The neuropil appears extremely loose with a large number of macrophages. Neurons which appear normal, can be seen on the left side (arrows). x 88. **B.** Higher magnification of small area of the anterior horn of the lumbosacral spinal cord 7 days after an IP injection of 6-AN. A neuron, Nu and macrophages (arrows) can be seen in the neuropil. x 1,360

Fig. 3. A. Anterior horn of the lumbosacral spinal cord 8 weeks after an IP injection of 6-AN. The neuropil appears compact. Neurons of various sizes can be seen (arrows). The small neurons in the area outlined appear to be less in number when compared to the corresponding region of Fig. 1A. x 88. **B.** An enlarged view of a small area from Fig. 3A showing the compact neuropil and a neuron (Nu). x 1,360

Fig. 4. A section of the lumbosacral spinal cord from a normal rat showing neurons labelled with FG. x 35

Fig. 5. FG labelled neurons in the lumbosacral spinal cord of a rat 7 days after an IP injection of 6-AN. x 35

were occasional small spots of haemorrhages (petechiae) but there was no evidence of infiltration of macrophages. 7 days after the injection of 6-AN, the gray matter appeared to be extremely oedematous with a large number of macrophages accumulated in the wide interstitial spaces (Fig. 2A). Some of the macrophages were lying adjacent to the blood vessels. Large normal neurons were observed in the ventrolateral portion of the anterior horns. The central portion of the anterior horns [laminae VI and VII of Rexed (1954)] showed a few small neurons and abundant macrophages (Fig. 2B). 8 weeks after the 6-AN injection, the gray matter appeared to be as compact as in the normal rats (Figs. 3A,B). The small neurons in laminae VI and VII of the anterior horns appeared to be fewer in number than in the normal spinal cords (compare Figs. 3A and 1A). Following an IP injection of FG, labelled neurons were observed bilaterally in the anterior horns of the lumbosacral spinal cord. The pattern of labelling both in terms of its intensity and number of labelled cells was comparable in both the normal and in rats given 6-AN injections (Figs. 4, 5). This was true in all the 6-AN injected rats sacrificed at various time intervals.

Routine electron microscopy

Randomly distributed microglial cells were observed in the anterior horns of the lumbosacral spinal cords of normal rats. Some of them were closely associated with neuronal soma. They displayed typical features of microglia, i.e. a small nucleus with dense chromatin masses, a variable number of lysosomes and long cisternae of rough endoplasmic reticulum (Fig. 6). 1 day after 6-AN injection, the cells showed some myelin-like inclusions in their cytoplasm indicating the activation of their phagocytic activity (Fig. 7). 7 days after 6-AN injection, extensive lesion occurred in the anterior horns of the lumbosacral spinal cords as evidenced by the accumulation of large number of macrophages. The latter showed a nucleus with dense chromatin clumps and abundant cytoplasm containing dense granules, vacuoles and phagosomes. Large chromatolytic neurons were observed among the massive macrophages. They appeared hypertrophic and round in shape with their nucleus displaced to the side. There was clumping of the rough endoplasmic reticulum (Fig. 8). Neurons of varying degree of degeneration occurred in laminae VI and VII of the anterior horns (Figs. 9, 10). The nuclear outline in these neurons was often irregular. In some cells, the cytoplasm was extremely «watery» due to the dispersion and disintegration of organelles (Fig. 9); in others, the cytoplasm was filled with closely packed cylindrical mitochondria (Fig. 10). Many degenerating axons were also observed in the same site (Fig. 11). In longer surviving animals, i.e. 8 weeks after the injection of 6-AN, all the surviving neurons appeared normal. Macrophages were rarely observed in the

Fig. 6. A microglia cell lying close to a neuron (Nu) in the lumbosacral spinal cord of a normal rat. The nucleus (N) of the microglial cell shows dense chromatin clumps. The cytoplasm shows some dense granules (circle) and cisternae of rough endoplasmic reticulum (arrows). x 6,000

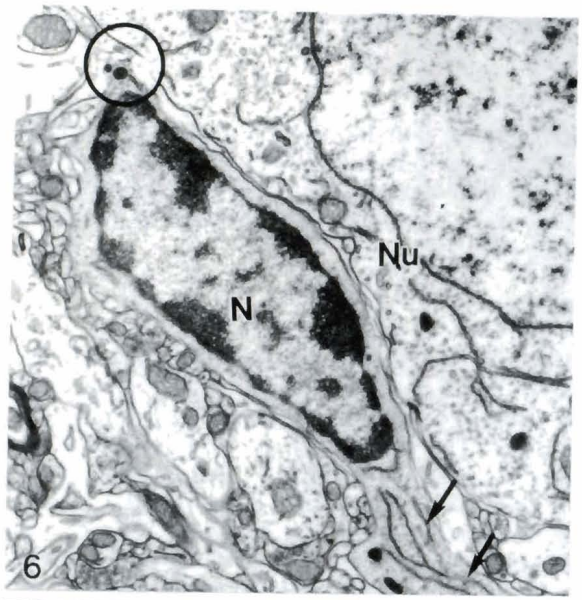
Fig. 7. An activated microglial cell in the lumbosacral spinal cord 1 day after an IP injection of 6-AN. The cell shows a nucleus (N) with dense chromatin clumps. The cytoplasm contains myelin-like inclusions (M). A Golgi apparatus (G) is present 4,950

Fig. 8. Portion of a large efferent neuron in the lumbosacral spinal cord 7 days after an IP injection of 6-AN. The nucleus (N) of the neuron has shifted peripherally. In the cytoplasm, the rough endoplasmic reticulum is present in small amounts and shows clumping which is indicative of neuronal chromatolysis. x 4,500

Fig. 9. A small neuron which appears to be degenerating in the lumbosacral spinal cord 7 days after an IP injection of 6-AN. The nucleus (N) is irregular. The cytoplasm appears watery with few organelles. x 6,600

Fig. 10. Portion of a degenerating neuron, in the lumbosacral spinal cord 7 days after an intraperitoneal injection of 6-AN. x 6,600

Fig. 11. A degenerating axon in the lumbosacral spinal cord 7 days after IP injection of 6-AN. x 7,000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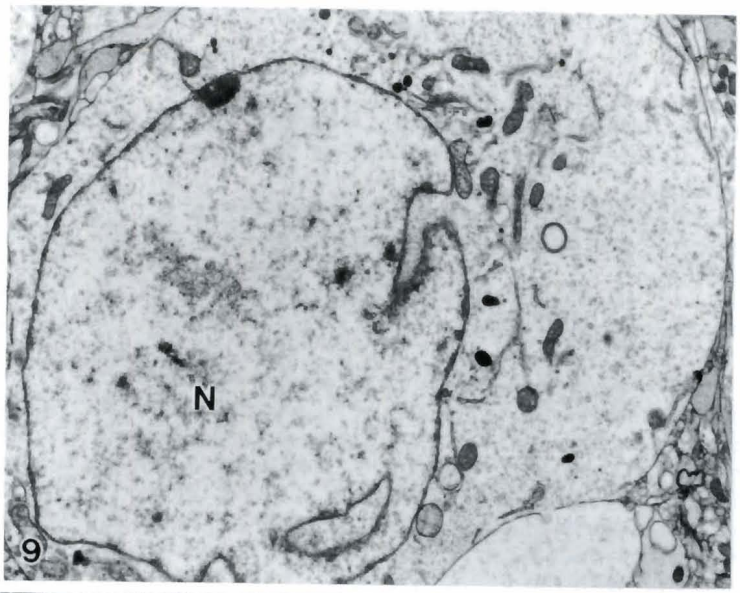
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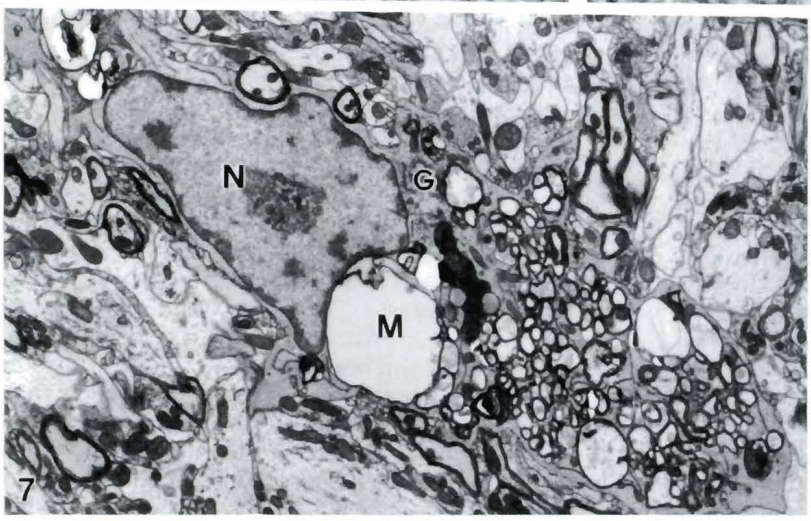
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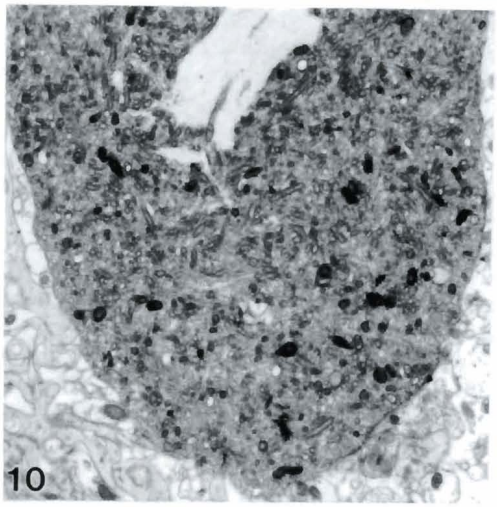
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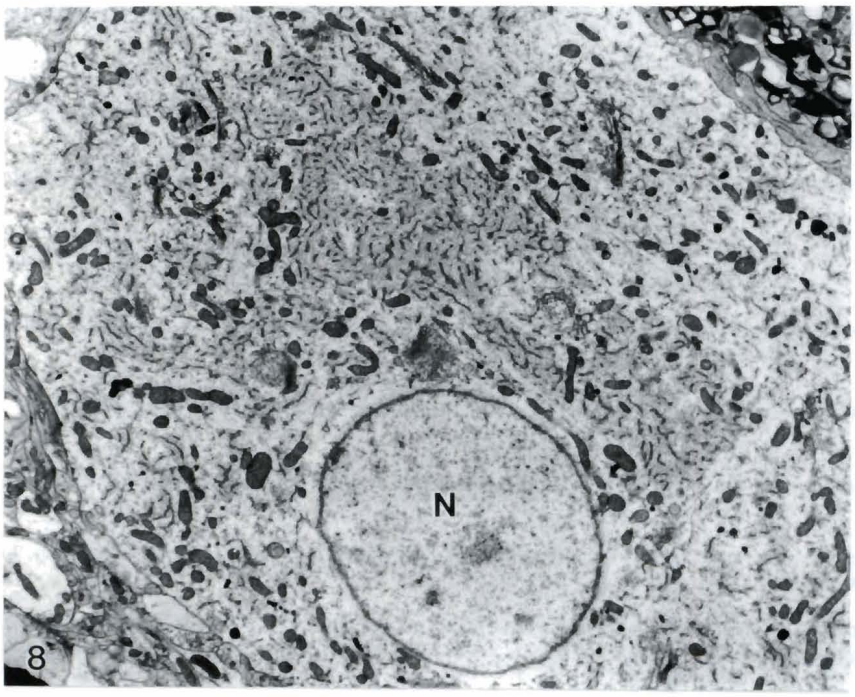
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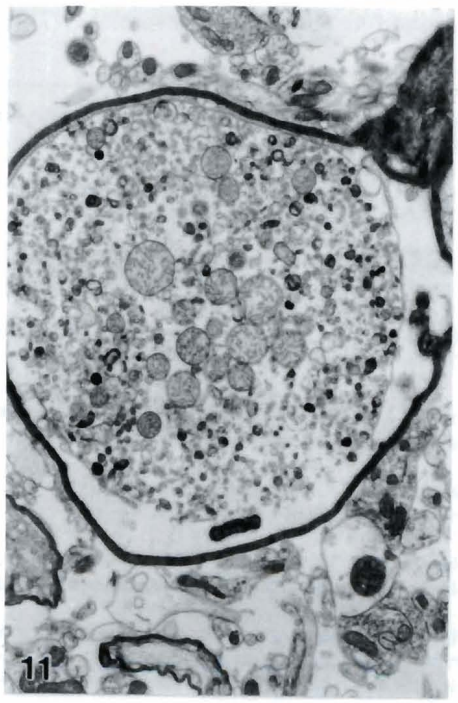
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animals with long survival times. A variable number of cells resembling typical microglia however were observed.

In all the 6-AN injected rats examined, there was no evidence of structural alteration at the neuromuscular junctions.

Immunohistochemistry

In the normal animals, immunoreactivity was barely detected in some microglial cells in the anterior horns of the lumbosacral spinal cord with the monoclonal antibody OX-42 (Fig. 12). With OX-18 or OX-6, none of the glial elements were stained. 6 hours after an intraperitoneal injection of 6-AN, immunoreactive cells were hardly observed in the anterior horns except when incubated with the antibody OX-42. 1 day after the 6-AN injection, a variable number of immunoreactive cells were observed with the monoclonal antibodies OX-42, OX-18 or OX-6. These cells appeared hypertrophic with short and thick processes (Fig. 13). 4-10 days after the injection, intense immunoreactivity was observed in the anterior horns (Fig. 14). This was localized in a large number of cells majority of which were round (Fig. 15). 2 weeks after the injection of the 6-AN, the number of immunoreactive cells was drastically reduced. While some of these immunoreactive cells maintained their round appearance at this post-injection interval, others assumed a ramified form (Figs. 16, 17). 3-4 weeks after 6-AN injection, the immunoreactivity in the anterior horn diminished and this was confined to some extremely branched cells (Fig. 18). 8 weeks after the injection, the immunoreactivity was comparable to that in the normal animals.

In immunoelectron microscopy of the material obtained at the peak of lesion, i.e. 7 days after 6-AN injection, intense immunoreaction was observed in the macrophages specifically localized at their plasma membrane and its invaginations and cytoplasmic vacuoles with the above mentioned monoclonal antibodies (Fig. 19).

Discussion

Following the administration of 6-AN, obvious chromatolytic changes occurred in some of the large efferent neurons in the anterior horns of the lumbosacral spinal cord. The structural alterations conformed in general with the description by Horita et al. (1978). With longer surviving times, however, the neurons regained their normal appearance. There were only sporadic degenerating neurons and these were mainly small cells. Neuronal loss resulting from 6-AN injection, if any, would be quite minimal. This is confirmed by the fact that the number of neurons labelled by FG in rats given 6-AN injection appeared to be comparable to that in the normal rats. Since FG is known to label CNS neurons that project to the peripheral target tissues (Leong and Ling, 1990) and since there was no obvious reduction in the number of labelled neurons in the 6-AN injected rats, the degenerating neurons observed in the present study would be primarily internuncial neurons whose processes remained in the anterior horns. This is compatible with the finding of Horita et al. (1978) that 6-AN causes the necrosis of internuncial cells in the laminae VI and VII. Thus, the degenerating axons observed in the present study would have originated from these cells. A striking feature of the anterior horns 1-10 days after the injection of 6-AN was the appearance of large number of macrophages. Other changes included the spongy vacuolation of the neuropil

Fig. 12. A microglial cell (arrow) in the anterior horn of the lumbosacral spinal cord from a normal rat showing faint immunoreactivity with the monoclonal antibody OX-42. x 340

Fig. 13. Activated and hypertrophied microglial cells showing intense immunoreactivity with the antibody OX-42 in the anterior horn of the lumbosacral spinal cord 1 day after an IP injection of 6-AN. x 340

Fig. 14. Anterior horn of the lumbosacral spinal cord 4 days after an IP injection of 6-AN. Intense immunoreactivity can be seen in the anterior horn with the antibody OX-18. Details of the immunoreactive cells cannot be made out at this magnification. Arrows indicate large efferent neurons. x 110

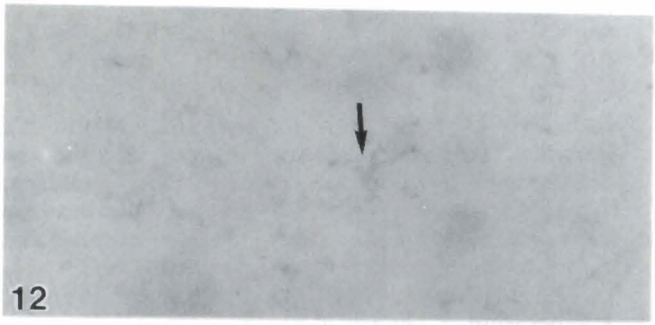
Fig. 15. A portion of the anterior horn of the lumbosacral spinal cord 10 days after an IP injection of 6-AN. A number of round cells showing immunoreaction with the antibody OX-42 can be seen. x 340

Fig. 16. A number of branched cells showing immunoreaction with the antibody OX-6 can be seen in the anterior horn of the lumbosacral spinal cord 2 weeks after an IP injection of 6-AN. x 340

Fig. 17. A few round cells (arrows) showing immunoreaction with the antibody OX-42 in the anterior horn of the lumbosacral spinal cord 2 weeks after an IP injection of 6-AN. x 340

Fig. 18. Some branched cells (arrow) resembling microglial cells, in the anterior horn of the lumbosacral spinal cord 4 weeks after an IP injection of 6-AN. The cells are weakly stained with the antibody OX-42. x 340

Fig. 19. an electron micrograph of a macrophage in the anterior horn of the lumbosacral spinal cord 7 days after an IP injection of 6-AN. The cytoplasm shows a Golgi apparatus (G) and a number of cytoplasmic inclusions. A positive reaction for the antibody OX-18 is localized on the plasma membrane (arrows) and in some of the cytoplasmic vacuoles (V). N, nucleus. x 12,000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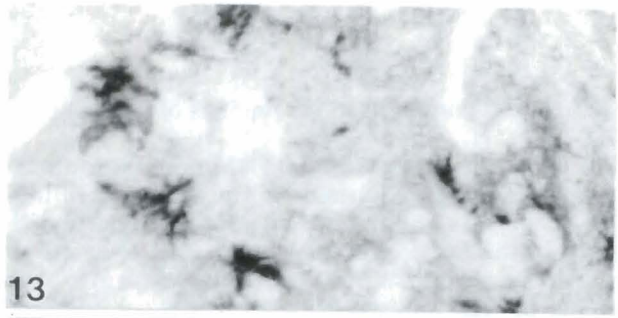
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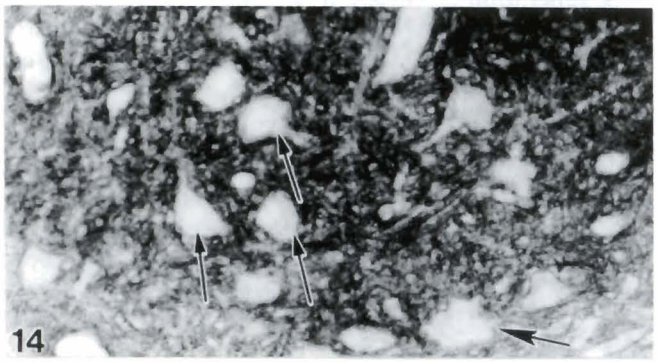
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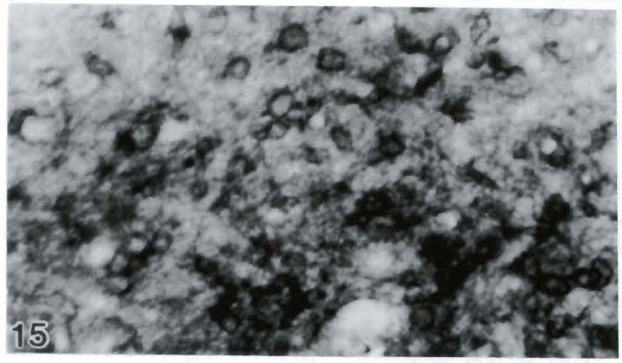
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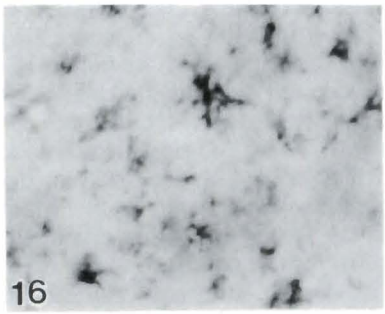
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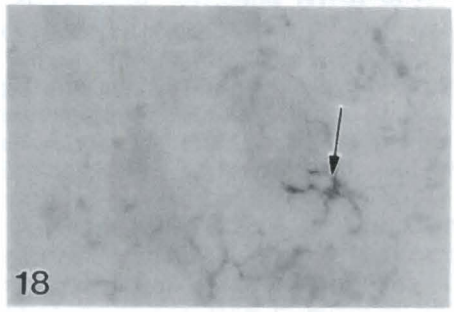
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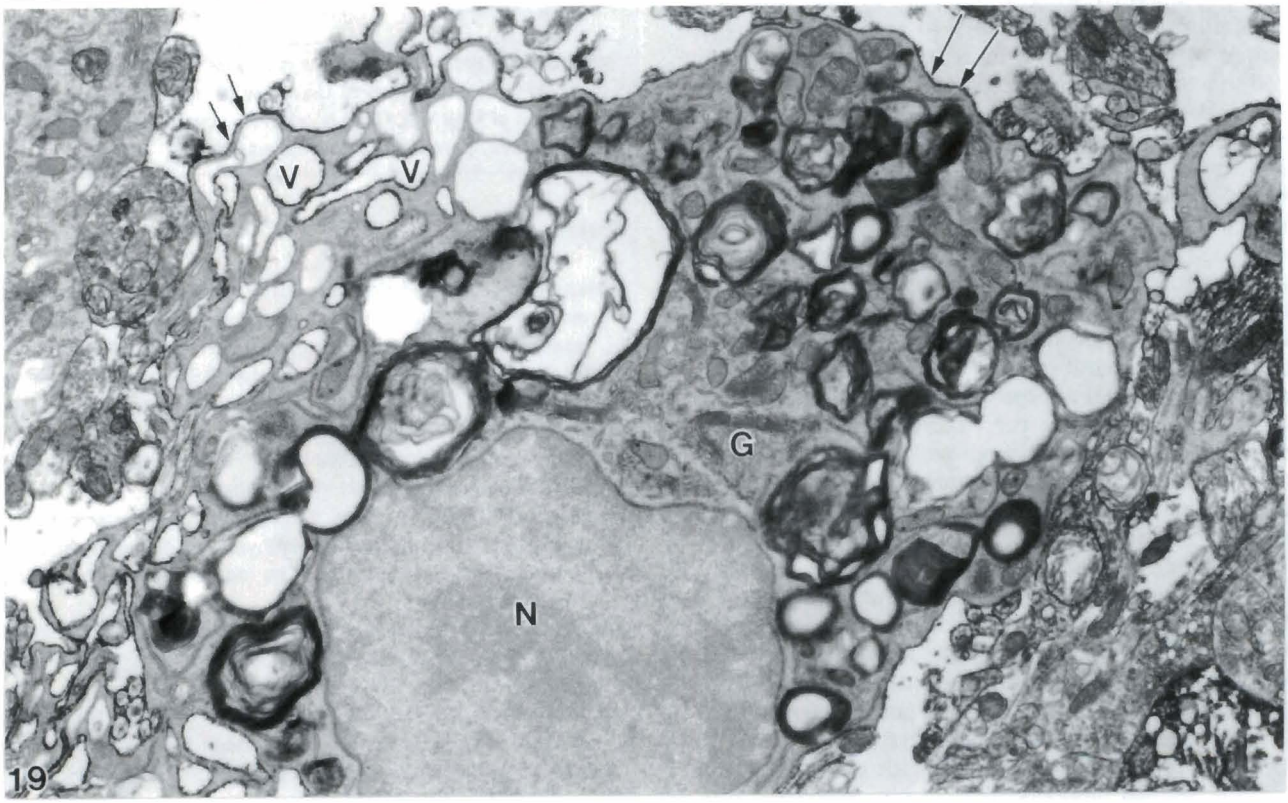

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a feature which corroborated the findings of Horita et al. (1978).

The present study showed that neurological symptoms ensuing 6-AN injection particularly in the limbs of the rats, coincided with the chromatolytic changes in the large efferent neurons of the anterior horns in the lumbosacral cord. Interestingly, the capability of their retrograde transport was not affected as shown by their uptake of FG. 2 weeks after the administration of 6-AN, the rats started to recover and by 4-8 weeks they regained their normal motor activity. The restoration of motor activity was clearly consistent with the normal feature of the large efferent neurons in the anterior horns in the longer surviving animals although there was a possible loss of some internuncial neurons.

Our present immunocytochemical study showed that with the monoclonal antibody OX-42 a large number of non-neuronal cells in the anterior horns of the lumbosacral spinal cord were intensely stained. 1 day after 6-AN injection, these cells appeared to be hypertrophic with thick branching processes. They were considered to be activated microglial cells because they shared all the features of microglial cells in the electron microscope. Furthermore, there was no indication of infiltration of blood elements within 1 day after 6-AN injection. It is known that microglia are morphologically inactive cells in normal nervous tissue but may be activated by a variety of inflammatory or degenerative stimuli (Cammermeyer, 1970; Torvik, 1972). In the present study, their activation was indicated by the presence of some inclusions in the cytoplasm. The activation could be due to the chromatolytic changes observed in the neurons since some of the microglia observed were closely apposed to neuronal somas. 4-10 days after the injection, most of the immunoreactive cells were round and intensely stained with the antibody OX-42. The large number of OX-42 positive cells were identified as macrophages observed in the electron microscope. They could be derived from activated microglial cells which became phagocytic as well as recruitment from blood monocytes. Previous studies have indicated that microglia and monocytes are the two main sources of macrophages in neural injuries (Marty et al., 1991; Kaur et al., 1992). At present, however, there is no satisfactory marker to differentiate the macrophages from these sources of origin. The monoclonal antibody OX-42 is known to mark type 3 complement receptors (CR3) in the plasma membrane of monocytes and their derivative macrophages (Beller et al., 1982; Wright et al., 1983). The presence of these receptors on the activated microglia and macrophages in the present study may be related to their active role in phagocytosis, since it has been shown that in monocytes and their derivative macrophages the receptors mediate endocytosis (Newman et al., 1980; Abrahamson and Fearson, 1983).

The activated microglia and macrophages in the present study were also stained with the monoclonal antibodies OX-18 and OX-6 at the various time intervals. These antibodies are known to mark the major histocompatibility complex class I (MHC I) and class II (MHC Ia) antigens which are undetectable in normal microglia. Their expression in activated microglia and macrophages in the present study is uncertain.

The primary role of macrophages in neural degeneration is clearly to remove the cellular debris. Consequently, they either degenerate in situ or transform into microglia (Kaur et al., 1987). In the present study, the macrophages displayed intense immunoreactivity for surface antigens 1-10 days after the injection of 6-AN. This, however, subsided with the survival time so that by 4 weeks after the injection of 6-AN only faint immunoreactivity was observed in some of the cells which appeared to be extremely branched resembling microglial cells. It is postulated that their phagocytic function probably had completed by this time and this had led to the down regulation of their surface antigens.

In conclusion, an intraperitoneal injection of 6-AN in rats resulted in the chromatolysis of some large efferent neurons in the anterior horns of the lumbosacral spinal cord and degeneration of internuncial cells as well as activation of microglial cells. Monocytes also infiltrated into the site of lesion giving rise to blood borne macrophages which were indistinguishable from the activated microglia. With time, some of the macrophages probably transform into microglia. Concomitant with this metamorphic change was a gradual diminution of the immunoreactivity of their surface antigens.

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