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Expression of major histocompatibility complex antigens and CR3 complement receptors in activated microglia following an injection of ricin into the sciatic nerve in rats

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Summary. The ventral horn motor neurons in the lower lumbar cord underwent rapid degeneration following an injection of *Ricinus communis* agglutinin-60 (RCA) into the sciatic nerve. The cell death which was most drastic between the fifth and seventh post-injection day elicited a significant increase in the number of microglia. The activated microglia were scattered throughout the neuropil but the dramatic feature was their close association with the somata of the degenerating neurons. Often several microglial cells were seen surrounding the soma of a degenerating neuron. Immunocytochemical study showed that both the interstitial as well as the perineuronal activated microglia were labelled with the monoclonal antibodies OX-18 and OX-42 for the detection of MHCI encoded antigen and type three complement receptors, respectively. Intense immunoreactivity was observed especially in the perineuronal microglia with OX-18. Electron microscopic study confirmed the identification of the activated microglia. Although the activated microglia closely apposed the neuronal soma, there was no sign of a direct endocytosis. The cytoplasm of the activated microglia, however, contained massive lipofuscin bodies in longer survival animals. Electron microscopic immunocytochemical study showed that the immunoreactivity of the activated microglia was localized along their plasma membrane facing the neuronal soma. Since the microglia cells on the contralateral side of the ventral horn were not marked by the antibodies used, it was postulated that the vigorous expression of MHCI antigen and CR3 receptors on the activated microglia was induced by the neuronal degeneration resulting from the application of the toxin ricin.

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Introduction

The adult central nervous system (CNS) is considered to be an immunologically priviliged site under normal conditions. This is because the molecules of the major histocompatibility complex (MHC) necessary for restricted antigen recognition are negligible or undetectable in the nervous tissue (Lampson and Fisher, 1984; Lampson and Hickey, 1986; Matsumoto and Fujiwara, 1986, 1987; Hickey and Kimura, 1987). However, in neurodegenerative disorders, e.g. multiple sclerosis (Woodroofe et al., 1986; Hayes et al., 1987), Alzheimer's disease (McGeer et al., 1987) and experimentally induced neurodegeneration (Akiyama et al., 1988; Akiyama and McGeer, 1989; Konno et al., 1989; Streit et al., 1989; Poltorak and Freed, 1989; Weinstein et al., 1990) MHC antigens have been detected on the microglial cells. While it may be true that the normal CNS in the adult is immunologically priviliged, the situation may not be quite the same in the developing brain in view of our recent study that the macrophagic amoeboid microglial cells in the postnatal rat brain show an intense immunoreactivity for MHCI antigen (Ling et al., 1991) and type 3 complement receptors (CR3) (Ling et al., 1990). The vigorous expression of MHCI encoded antigen on amoeboid microglia which have been considered to be the precursor of microglia in the adult CNS indicates the putative role of these cells to interact with T-cytotoxic/supressor cells (Ling et al., 1991). Our studies also showed that the expression of MHCI and CR3 on these cells gradually decline with age so that by about the weaning age (21-23 days old), the immunoreactivity was extremely weak and was barely detected occasionally on a few ramified microglial cells (Ling et al., 1991).

The aim of the present study sought to ascertain if the surface antigens on the ramified microglia, a glial type that persists through the adult may be activated under experimental conditions. A model of choice for this purpose would be the efferent neurons in the lower lumbar cord projecting to the sciatic nerve because our earlier study (Ling et al., 1989a) had already established the fact that these neurons were selectively destroyed by their «suicidal» transport of *Ricinus communis* agglutinin (RCA) introduced into the sciatic nerve. The advantage of this method is that there was no evidence of infiltration of mononuclear cells into the site of neural degeneration which would otherwise complicate the issue of the origin of neural macrophages. With this model, it may be assumed then that the reactive microglia at or in the vicinity of neural degeneration are derived exclusively from the activation of endogenous microglia.

Materials and methods

Male Wistar rats weighing about 250 g were used in this study. Under 7% chloral hydrate anesthesia the right sciatic nerve was exposed following the separation of the gluteal maximus muscle. 1.5 µl of 0.05% *Ricinus communis* agglutinin-60 (RCA-60) (Lot No. P83X02, Seikagaku Kogyo Co Ltd., Japan) in 0.01M phosphate buffer was injected into the nerve with a Hamilton syringe. After the injection the nerve was crushed for 5 seconds just proximal to the site of injection with a pair of artery forceps. The animals were allowed to survive for 5, 6, 7, 12 and 21 days before they were sacrificed.

For light microscopy, the animals were re-anesthetized and perfused with 10% neutral formalin. The spinal cord extending from the mid-lumbar to upper sacral segment was removed and processed. Transverse serial sections at 7 µm were cut and stained with cresyl fast violet.

For routine electron microscopy, the animals were perfused with a mixed aldehyde solution composed of 2% paraformaldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4. The perfusion lasted for about 30 minutes. The spinal cord was removed and further fixed in a similar fixative for an additional 2 hours. The tissue was then rinsed in several changes of sucrose buffer. Vibratome sections of 80 µm thickness were prepared and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour. The tissue sections were then dehydrated and embedded in Araldite mixture. Ultrathin sections were double stained with uranyl acetate and lead citrate and were examined in a JEOL 1200 EX electron microscope.

For light microscopic immunocytochemistry, the rats were perfused with Ringer's solution until the liver was clear of blood. This was followed by the 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 15-20 minutes after which the spinal cord extending from the lumbar to the upper sacral region was removed and fixed in a similar fixative for 2 hours. The spinal cord was then kept in 0.1 M phosphate buffer containing 10% sucrose overnight at 4° C. 40 µm frozen sections were cut and rinsed in phosphate buffer saline (PBS). The sections were then incubated with the monoclonal antibodies OX-18 and OX-42 (Sera Lab MAS 101b and MAS 370b) diluted 1:100 with PBS for 18 hours. Vectastain ABC-kit (PK-4002, Vector Laboratories) against mouse IgG was used for the subsequent detection of antibodies with 3,3'-diaminobenzidine as a peroxidase substrate.

For electron microscopic immunocytochemistry, the rats were perfused with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer for 15 minutes followed by a second fixative composed of a mixture of periodate-lysine-paraformaldehyde (McLean and Nakane, 1974) for 15 minutes. After perfusion the spinal cord was removed as for light microscopy and kept in 0.1 M phosphate buffer containing 10% sucrose at 4° C. 50 µm vibratome sections were cut and incubated for immunocyto-chemical reaction as above for light microscopy. The sections were then post-omicated for 30 minutes in 1% osmium tetroxide in 0.1 M phosphate buffer. The sections were then dehydrated in a graded series of alcohol and embedded in Araldite mixture. Ultrathin sections were stained with lead citrate only and were examined in a JEOL 1200 EX electron microscope.

Results

Light Microscopy

As reported in our earlier study (Ling et al., 1989a), injection of RCA-60 into the sciatic nerve resulted in a rapid and selective destruction of the motor neurons especially those localized at the lateral part of the ventral horn in the lower lumbar spinal cord (Fig. 1). The neurons on the contralateral side were unaffected by the injection. Neuronal death was most drastic between the fifth and seventh postoperative days. A remarkable degenerative change of the neurons was the hypertrophy and reduced density of their somata (Fig. 2) when compared with the neurons on the contralateral side. Lysis of the neurons occurred with a concomitant glial reaction.

Immunocytochemistry

With the monoclonal antibody OX-18, intense immunoreactivity was observed at the site of neuronal degeneration 6 days after the RCA injection (Fig. 3). The contralateral site was not stained. The immunoreactivity on the injected side was localized on the microglial cells encircling the neuronal somata (Figs. 4-6). In the neuropil area between the neuronal somata, there were many positive cells some of which were enlogated and ramified (Figs. 4, 7). Fig. 7 also

depicts RCA-poisoned neurons at various stages of degeneration. In some the outline of the neuron and its nucleus was still discernible (Fig. 7).

With the monoclonal antibody OX-42, immunoreactivity was also visualized in the cells surrounding the degenerating neurons (Fig. 8). Neurons not affected by RCA were not associated with any OX-42 positive cells (Fig. 9). When compared with OX-18, there were relatively fewer OX-42 positive cells in the neuropil area.

Electron Microscopy

Electron microscopic study confirmed the above findings. Microglial cells responded to the neuronal degeneration. Sometimes as many as up to six microglial cells in a section profile surrounded the soma of a degenerating neuron (Fig. 10). In a more advanced stage of degeneration, an almost empty space probably resulted from the dissolution of neuron was surrounded by several microglial cells (Fig. 11). The cytoplasm of these microglial cells was often filled with large masses of lipofuscin of heterogeneous texture (Fig. 11). On closer examination, the cytoplasm of the activated microglial cells showed a well-developed Golgi apparatus, a variable number of lysosomes and isolated profiles of rough endoplasmic reticulum (Fig. 12). The plasma membrane of microglial cells was closely apposed to the neuronal soma with no distinguishable membrane (Fig. 12). A few filipodial and pseudopodial processes sometimes projected from the plasma membrane of the microglial cells facing the neuropil.

The present electron microscopic study confirmed the identification of the OX-18 positive cells surrounding the somata of the degenerating neurons. The positive cells identified as microglia showed immunoreactivity along its plasma membrane apposed to the neuron (Fig. 13). Weak immunoreactivity was observed along the rest of the plasma membrane facing the neuropil (Fig. 13). The OX-18 positive microglia contained a nucleus with margination of chromatin clumps (Fig. 13). The cytoplasm contained the usual organelles including some lipid droplets (Fig. 13).

Discussion

Following an injection of RCA into the sciatic nerve, the number of microglial cells in the ventral horn of the lower lumbar cord was drastically increased so that by the seventh postoperative day the value was more than three times (38%) that of the normal or the contralateral (11%) side (Ling et al.,

Fig. 1. Transverse section of lower lumbar spinal cord 6 days after a single injection of RCA into the right sciatic nerve. Note the destruction of a group of motor neurons in the ventral horn (VH). A few neurons, however, survive the injection of RCA (arrows). They are probably neurons projecting to the peroneal division of the sciatic nerve. C, central canal. Cresyl fast violet. x 40

Fig. 2. Selective destruction of motor neurons in the ventral horn of the lower lumbar cord 6 days after RCA injection. The degenerating neurons are hypertrophied (arrows). Gliosis is evident in the area circled. x 112

Fig. 3. Intense immunoreactivity is observed in the area outlined with the monoclonal antibody OX-18. The area shown corresponds to that in Fig. 1. 6 days after RCA injection into the sciatic nerve. C, central canal. × 49

Fig. 4. Showing the ventral horn of the lumbar cord 6 days after RCA injection into the sciatic nerve. Arrows indicate a few RCA-affected neurons. A large number of OX-18 positive cells are observed in the neuropil area (circle). × 194

Fig. 5. A close examination of one of the RCA-affected neurons shown in Fig. 4. This particular RCA-affected neuron is clearly hypertrophied with a pale nucleus (asterisk). OX-18 positive cells surround the soma of the neuron whose outline is still evident. Many ramified OX-18 positive cells are in the vicinity. 6 days after RCA injection. × 440

Fig. 6. A higher magnification of two RCA-poisoned neurons (asterisks) shown in Fig. 4. Lysis of the neurons is indicated by the dissolution of the cytoplasm especially the lower left cell which has a foamy cytoplasm. Intense immunoreactive cells (OX-18 positive) surround the degenerating cells. 6 days after RCA injection. × 440

Fig. 7. A group of four RCA-poisoned neurons (asterisk) undergo different degrees of degeneration. They are surrounded by a variable number of OX-18 positive cells. The nucleus and its containing nucleolus is still identifiable in the top most cell. The lowermost cell shows probably a more advanced stage of degeneration. The cells associated with its soma show an intense immunoreactivity. Arrows indicate ramified OX-18 cells in the neuropil. 6 days after RCA injection. × 270

Fig. 8. Two ventral horn neurons at advanced stage of degeneration (asterisks). OX-42 positive cells surround their somata. 6 days after RCA injection. × 270

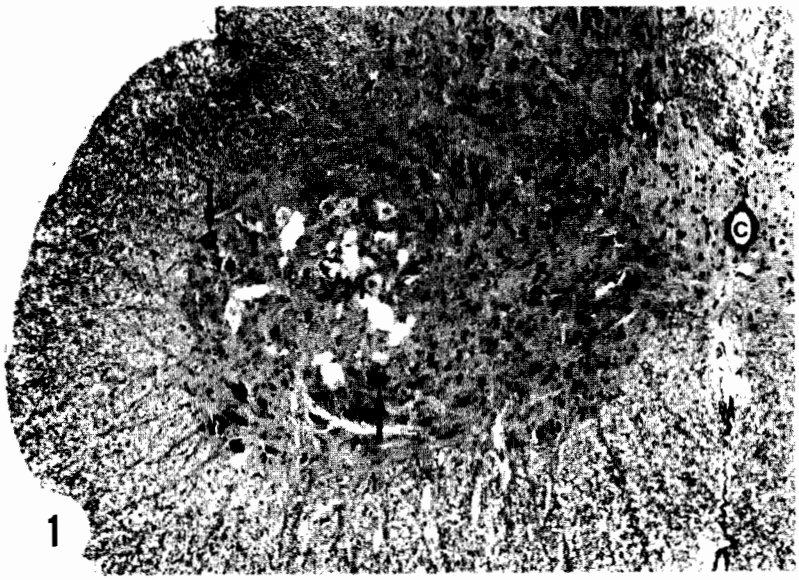
Fig. 9. A degenerating neuron (asterisk) in the ventral horn. Perineuronal cells are intensely stained with the monoclonal antibody OX-42. Arrows indicate two neurons that survive the RCA injection. Note the absence of OX-42 positive cells associating with their somata. 6 days after RCA injection. × 270

Fig. 10. Electron micrograph showing a RCA-poisoned neuron (N) in the ventral horn. It is surrounded by at least six microglial cells (m) with their long extending processes. The cytoplasm of the degenerating neuron is extremely vacuolated. 7 days after RCA injection. × 2,625

Fig. 11. Three microglial cells encircle a «cell ghost» most probably derived from a degenerated neuron poisoned by RCA given three weeks before. The microglial cells show masses of lipofuscin granules (L). × 3,500

Fig. 12. A portion of an activated microglia in close approximation to a degenerating neuron (DN) containing swollen mitochondria (M) with disrupted cristae. The cytoplasm of the microglial cell shows a well-developed Golgi apparatus (G), cisternae of rough endoplasmic reticulum (rER) and lysosomes (Ly). N, nucleus of microglia. 7 days after RCA injection. × 21,000

Fig. 13. Two activated microglia (M1, M2) appose a degenerating neuron containing some debris following the disintegration of the neuronal soma (asterisk). Strong immunoreactive reaction with OX-18 is seen along the plasma membrane facing the neuron (arrows). Elsewhere along the plasma membrane of the microglial cells the immunoreactivity is rather weak (open arrows). L, lipid droplets. 6 days after RCA injection. × 14,000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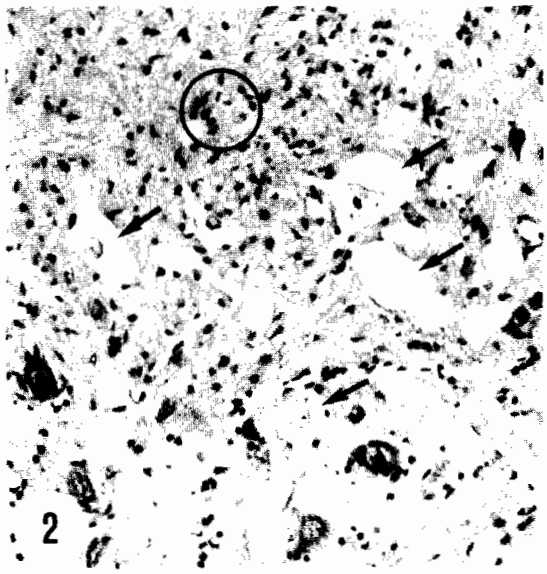
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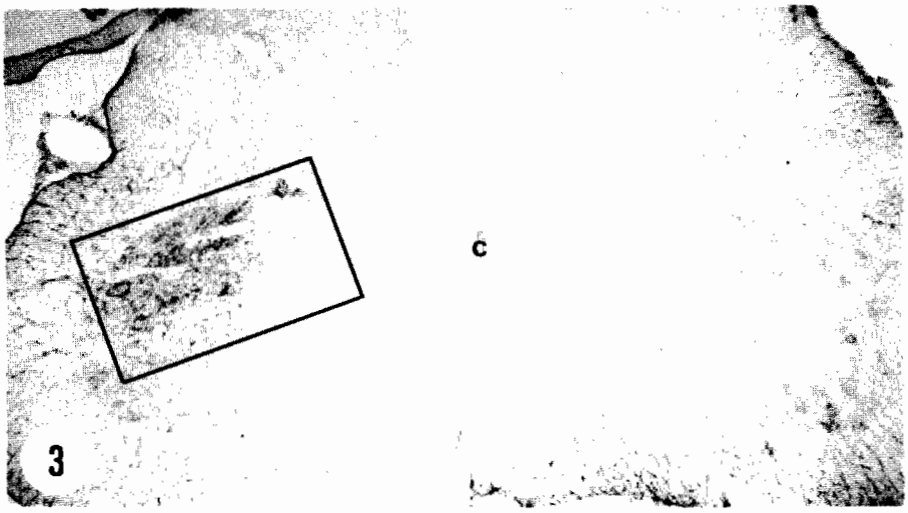
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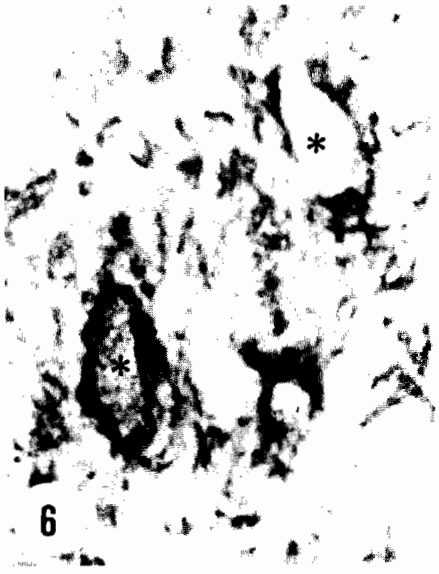
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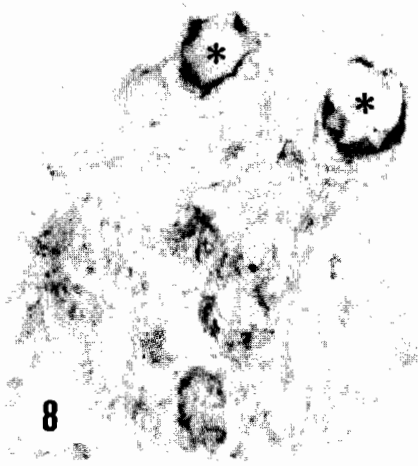
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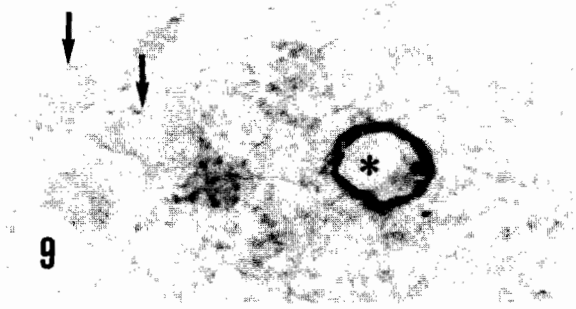
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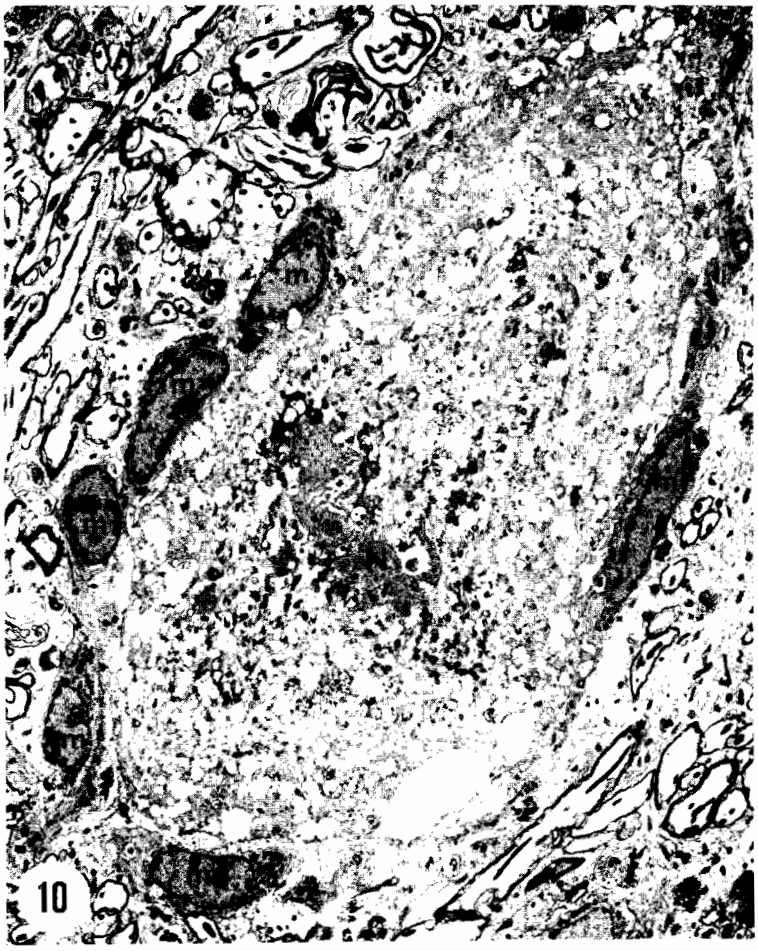
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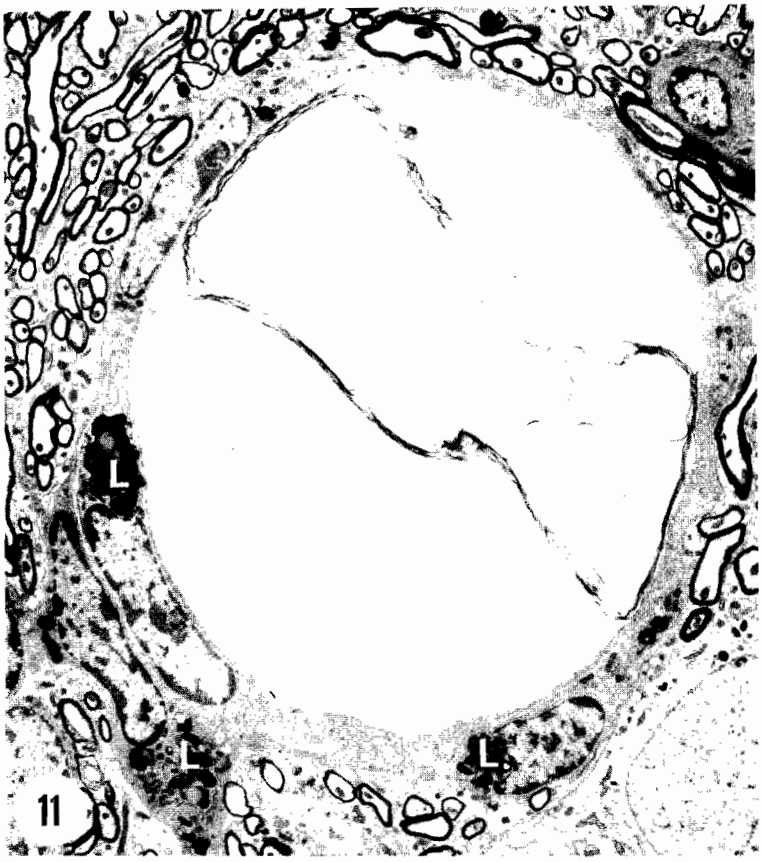
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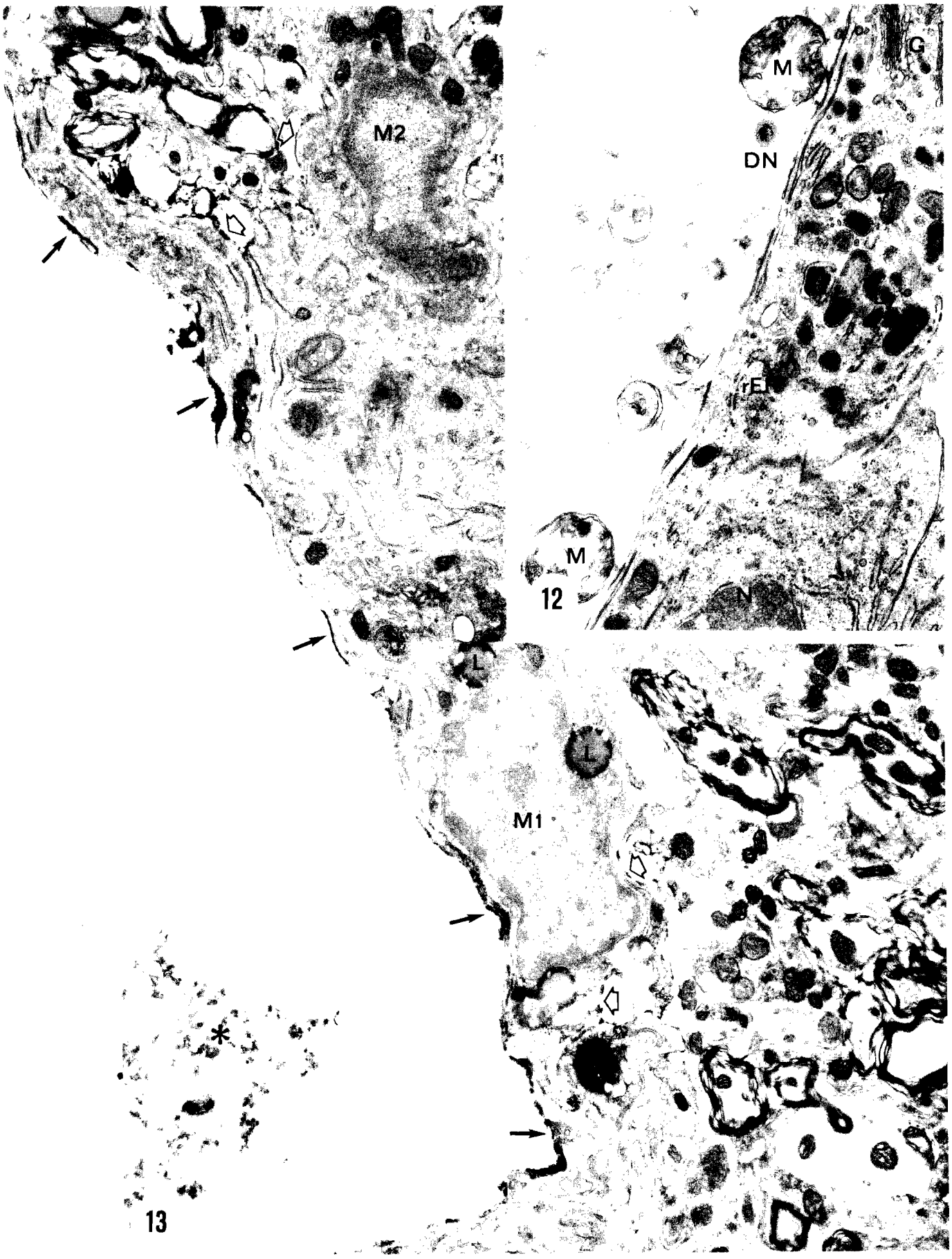
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1989a). The population of oligodendrocytes and astrocytes remained relatively unchanged over the same period. The upsurge of microglial cells is therefore a clear evidence of response to the rapid neuronal death caused by the RCA. The mechanism that induces their proliferative activity is only speculative. One possible explanation would be that some specific substances might have been released by the RCA-poisoned neurons that attracted the proliferation and migration of microglia (chemotaxis). It may even be possible that the RCA taken up earlier by the neurons may be released into the neuropil causing the activation of the endogenous microglia. A behavioural response of the microglial cells was their close association with the somata of dying neurons. This was confirmed by our present electron microscopic observation. Several perineuronal microglia presumably already activated closely espoused the neuronal soma.

Our present immunocytochemical study demonstrated the expression of MHCI encoded antigen and CR3 receptors on the activated microglial cells in response to the neuronal death caused by RCA. More intense immunoreactivy was observed in the perineuronal microglia than those in the neuropil suggesting a higher level of surface antigens and receptors was raised in these cells. Since the microglial cells on the contralateral side did not show a similar immunoreactivity, it can be confidently deduced that the expression of these surface antigens was triggered off by the neuronal death. The immunophenotypic expression of these cells is evidently not a feature acquired *de novo* since our earlier studies (Ling et al., 1990, 1991) had already demonstrated a similar immunoreactivity on their precursor cell, i.e. amoeboid microglia in the postnatal period. It was described that towards the latter part of the postnatal period when the cells transformed into the ramified microglia the immunoreactivity gradually diminished. The diminution is probably due to their reduced phagocytic activity whence the cells enter a dormant form which persists through the adult CNS. It is likely that the surface antigens and receptors are masked or suppressed during this period and this might explain the failure by some authors to detect any monocytic membrane antigens and mononuclear phagocyte markers on microglia (Oehmichen et al., 1979; Wood et al., 1979; Tsuchihashi et al., 1981).

The present study confirmed the findings of Streit et al. (1989) who injected the ricin into the facial nerve and showed the expression of MHC antigens on microglia in the facial motor nucleus. The immunoreactivity as demonstrated by these authors, however, appeared rather weak on the perineuronal microglia probably because the amount of RCA given by these authors (1 mg/ml) was insufficient to induce a rapid neuronal degeneration.

The significance of the re-expression of MHCI antigens and CR3 receptors on activated microglia is only speculative. In the developing brain their presence on amoeboid microglia was described to be related to the endocytotic activity. In the present study the RCA-poisoned neurons clearly underwent lysis. The activated microglial cells closely apposed the neurons but there was no sign of a direct phagocytosis. The presence of massive lipofuscin granules, on the other hand, suggests that the cells had ingested some substances earlier probably through CR3 mediated endocytosis of fluid phase materials. The intense immunoreactivity of the plasma membrane lining the neuronal soma with OX-18 indicates the concentration of MHC class I antigens at this site. It is known that MHC class I antigens serve as the restriction elements for cytotoxic/suppressor lymphocytes (Sell, 1987; Stites et al., 1987; Akiyama et al., 1988; Weinstein et al., 1990). It is therefore likely that following the exposure to the RCA probably released by the dying neurons, the activated microglial cells are ready to interact with T-cytotoxic/suppressor cells. There was no evidence in the present as well as in the earlier study (Ling et al., 1989a) of any infiltration of lymphocytes into the site of neuronal degeneration. Massive influx of mononuclear cells including lymphocytes, however, was observed in the hypoglossal nucleus following an intraneural injection of the toxic ricin into the hypoglossal nerve (Ling et al., 1989b). The results of the present study together with our earlier findings (Ling et al., 1991) show that microglial cells in the central nervous system in their embryonic form as amoeboid microglia and the reactive form in neural degeneration have the capability to interact with lymphocytes. This is probably a property inherent from their predecessor cells, namely, the circulating monocyte which had entered the CNS in the embryonic period (Ling, 1981).

Another feature which is worthy of consideration is that while the activated microglia in the CNS may revert to their embryonic form with respect to their immunophenotypic features, their external morphology, however, remained relatively unaltered as shown in the present electron microscopic study. It would seem that the activated microglia in other neurodegenerative changes (Akiyama et al., 1988; Akiyama and McGeer, 1989; Perlmutter et al., 1990; Weinstein et al., 1990) and neural transplantation (Poltorak and Freed, 1989) also maintained their ramified form. The failure of the activated microglia to revert to the round and amoeboid form as the precursor cells may be due to the limited spaces available in which the neural tissue is more closely packed.

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