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Lectin binding by resting and reactive microglia

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Summary

Conjugates of the B_4 isolectin from *Griffonia simplicifolia* seeds and horseradish peroxidase were used as a histochemical reagent for the specific visualization of microglial cells in the rat CNS. Resident microglia bearing galactose-containing glycoconjugates were stained throughout the brainstem and cerebellum. In the first week following axotomy of the facial nerve, a profound and rapid accumulation of reactive microglia, as evidenced by increasing lectin reactivity, was seen to take place in the facial nucleus. Light microscopy of paraffin sections demonstrated binding of lectin–horseradish peroxidase conjugates to microglial cytoplasmic processes. When ultrastructural cytochemistry was performed, reaction product was found localized on microglial plasma membranes, as well as on intracytoplasmic membranes. The glial reaction to axotomy was studied further with double labelling of microglia and astrocytes by lectin histochemistry and immunostaining for glial fibrillary acidic protein, respectively. Our results demonstrate the presence of membrane-associated glycoconjugates containing terminal α-D-galactose residues on microglia, but not on other glial cell types. The possible nature and function of these glycoconjugates are discussed.

Introduction

Studies concerned with differentiating between various glial cell types under normal and pathologic conditions have used a variety of histochemical methods including silver impregnation techniques (Del Rio-Hortega, 1965; Ibrahim *et al.*, 1974), enzyme histochemistry (Ibrahim *et al.*, 1974; Kreutzberg & Barron, 1978; Murabe & Sano, 1981) and, more recently, immunohistochemical approaches (Perry *et al.*, 1985; Esiri & McGee, 1986; Graeber & Kreutzberg, 1986; Schiffer *et al.*, 1986). While the silver carbonate method of Del Rio-Hortega allows reasonable distinction between micro- and macroglia at the light microscopic level, it is of limited value for electron microscopy because of stringent fixation requirements that do not allow good ultrastructural preservation (Mori & Leblond, 1969). Enzyme cytochemistry, on the other hand, permits staining for both light and electron microscopy. However, enzyme activities may not be confined to a specific glial cell type and are often found associated with glial membranes of micro- and macroglia, as shown for 5'nucleotidase (Kreutzberg *et al.*, 1978; Kreutzberg & Barron, 1978). With the discovery of glial fibrillary acidic protein (GFAP), a first immunocytochemical reagent suitable for the specific visualization of astrocytes became available (Bignami *et al.*, 1972). Subsequently, a number of studies have described the use of various monoclonal antibodies for the differential localization of oligodendroglia (Ghandour *et al.*, 1979), as well as microglia (Perry *et al.*, 1985; Esiri & McGee, 1986).

The phenomenon of glial cell proliferation in the CNS as a result of peripheral nerve damage has been described repeatedly, and has served to emphasize intercellular communication and functional interdependence of glial cells and neurons during pathologic conditions (Nissl, 1894; Cammermeyer, 1965; Sjöstrand, 1965; Kreutzberg, 1966; Blinzinger & Kreutzberg, 1968; Sumner, 1974; Torvik & Soreide, 1975; Aldskogius, 1982). Recently, GFAP staining has been employed successfully in this laboratory for the study of reactive astrocytes during the axonal reaction in the rat facial nucleus (Graeber & Kreutzberg, 1986).

The purpose of the present communication was to re-examine the glial reaction following axotomy from the viewpoint of carbohydrate histochemistry by using lectins labelled with horseradish peroxidase (HRP). This study was stimulated by previous findings demonstrating selective staining of a population of glial cells with the *Griffonia simplicifolia* B_4 isolectin (GSA I-B_4–HRP) in rat spinal cord (Streit *et al.*, 1985b). GSA I-B_4 has an exclusive sugar binding specificity for terminal α-D-galactose residues (Hayes & Goldstein, 1974). The glial cell population which demonstrated affinity for this lectin was tentatively identified as microglia based on morphologic criteria seen light microscopically. In the experiments described here we have used GSA I-B_4–HRP conjugates to label proliferating glia in the facial nucleus and

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have confirmed specificity of lectin binding to microglia by ultrastructural cytochemistry. In addition, a double labelling method employing GSA I-B_4–HRP in conjunction with GFAP immunostaining is presented, demonstrating simultaneous visualization of microglia and astrocytes in the same section.

Methods

Surgery

For all experiments adult male Wistar rats weighing 200–250 g were used. Under ether anaesthesia the right facial nerve was exposed and transected approximately 3 mm distal to the stylomastoid foramen.

Tissue collection and fixation

For light microscopy, animals were sacrificed at 1, 2, 3, 4, 6, 8, 11, 14, 17, 21 and 28 days after nerve section by cardiac perfusion with 50 ml of physiologic saline followed by 250 ml of a 2% solution of $HgCl_2$ in 1.25% sodium acetate buffer containing 0.5% glutaraldehyde, pH 6.1. This fixative was known from previous studies to allow optimal lectin reactivity (Streit *et al.*, 1985a, b). Following perfusion, the tissue was fixed for an additional 16 to 18 h by immersion in the same fixative at 4° C. Fixation was terminated by washing the tissue several times in 70% ethanol, and a slice of the brainstem, 0.5 cm thick, containing the facial nucleus was removed. The tissue was then dehydrated in ascending ethanols, cleared in xylene and embedded in paraffin. Sections were cut at 6 µm and treated with Lugol's iodide to remove mercury prior to staining.

For electron microscopy animals were sacrificed at 4 and 5 days after axotomy by perfusion via the left ventricle with 50 ml of saline followed by 250 ml of 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2. The brainstem was removed and fixed overnight in the same fixative at 4° C, and then washed in 0.1 M cacodylate containing 7.5% sucrose. Vibratome sections were cut at 60 µm and processed for lectin staining.

Staining methods

Purified isolectin B_4 from *Griffonia simplicifolia* seeds (GSA I-B_4) and HRP, type VI, were obtained from Sigma Chemical Co., and conjugates were prepared according to a one-step procedure (Avrameas, 1969). Briefly, 1.0 mg of lectin and 5.0 mg of HRP were dissolved in 0.5 ml of phosphate-buffered saline (PBS) at pH 6.8. To this were added 20.0 µl of a 1% aqueous glutaraldehyde solution, and the mixture was stirred for 2–2.5 h at room temperature. The sample was then applied to a Sephadex G-200 column and eluted with 0.1 M PBS, pH 7.2. Optical density was measured at 280 and 403 nm for each 2 ml fraction collected, and selected fractions to be used for staining were pooled and stored in aliquots at −20° C.

Following deparaffinizing and hydrating, sections were incubated in PBS containing cations (0.1 mM of $CaCl_2$, $MgCl_2$, and $MnCl_2$) for at least 10 min prior to application of lectin–HRP conjugates. Aliquots of the conjugates were diluted 1:10 in PBS containing cations and 0.1% Triton X-100. The slides were incubated in a moist chamber overnight at 4° C and then washed with PBS. Sites containing bound lectin–HRP conjugates were visualized using 3,3'-diaminobenzidine (DAB)–H_2O_2 substrate medium (Graham & Karnovsky, 1966). For double-labelling experiments the slides were incubated in 10% methanol and 0.3% H_2O_2 for 20 min after the DAB reaction to quench any remaining peroxidase activity. Following several washes in PBS, a mouse monoclonal antibody (Labsystems, Helsinki, Finland) to GFAP, diluted 1:400 in PBS, was applied for 2 h at room temperature. GFAP reactivity was localized using the avidin–biotin method (Vectastain ABC-Kit, Vector Laboratories, Burlington, CA), and using DAB+Co^{2+} ions as a substrate for peroxidase (Adams, 1981). This resulted in a bluish-black colouration of astrocytes which made them easily distinguishable from the brown lectin-stained microglia. Selected sections were counterstained with Cresyl echt violet or Methyl green.

Controls for lectin staining consisted of (a) treating sections prior to staining with α-galactosidase from coffee beans (Boehringer Mannheim) at 1 U ml^{-1} in phosphate–citrate buffer, pH 5.1, for 2 h at 37° C; and (b) incubating with GSA I-B_4–HRP in the presence of 0.1 M melibiose (6-0-α-D-galactopyranosyl-D-glucose) to saturate lectin binding sites and prevent interaction with sugars of tissue components.

As a control for GFAP reactivity the primary antiserum was replaced by PBS and the staining sequence carried out as before.

For the ultrastructural visualization of lectin binding sites, vibratome sections were first incubated in an aqueous solution of 0.1% trypsin (Sigma, type XI) and 0.1% $CaCl_2$, pH 7.8, at 37° C for 30 min, washed in several changes of PBS, and then incubated with GSA I-B_4–HRP diluted 1:10 in PBS containing cations and 0.1% Triton X-100 overnight at 4° C with gentle agitation. Reactive sites were visualized with DAB, and the tissue was postfixed in OsO_4, dehydrated, and embedded in Epon. Ultrathin sections were examined in a Zeiss EM 10 electron microscope after staining with uranyl acetate and lead citrate.

Results

Light microscopy of lectin binding

A population of glial cells throughout the brainstem and cerebellum was labelled with the GSA I-B_4–HRP conjugate, but not with other conjugates of galactose-specific lectins, such as peanut agglutinin and *Ricinus communis* agglutinin. These glial cells were characterized by small dense, sometimes triangular nuclei and numerous long undulating cytoplasmic extensions which contained the lectin reactivity (Fig. 1). The glial cells were seen to be distributed about equally in grey and white matter, and their overall morphology and distribution was consistent with descriptions of microglia using other staining methods. Specificity of GSA I-B_4–HRP for terminal α-D-galactose residues was confirmed by treatment of sections with α-galactosidase prior to application of the conjugate, and by incubating with the lectin in the presence of 0.1 M melibiose. Both of these control

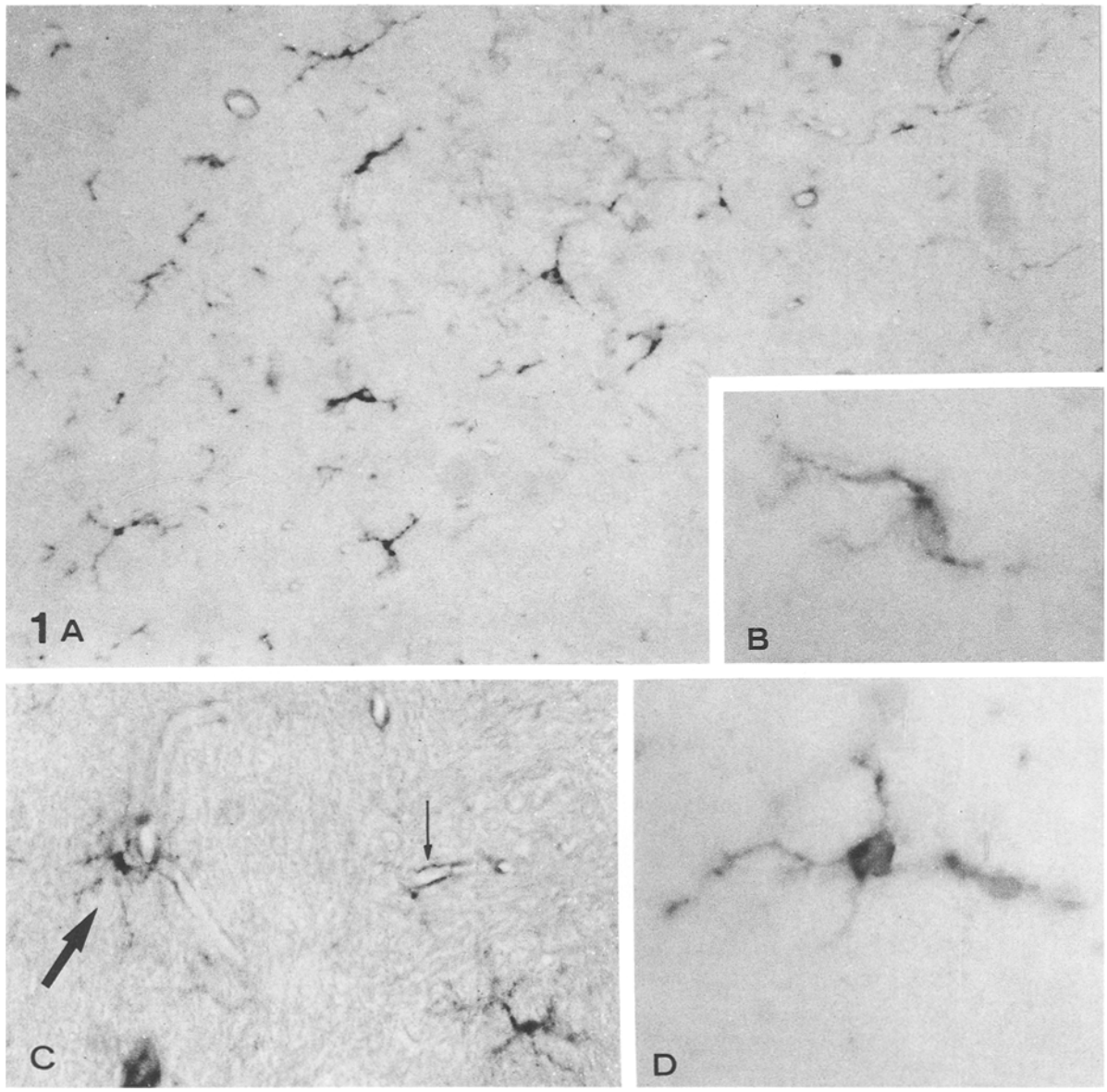


Fig. 1. Visualization of resident microglia with GSA I-B_4–HRP in normal rat CNS tissue. (A) Molecular layer of the cerebellum, × 320. (B) Single microglial cell in the cerebellum, Methyl green counterstain, × 800. (C) Cochlear nucleus. A microglial cell is seen in close proximity to a capillary (thick arrow). Note reaction product present also on capillary endothelium (thin arrow), × 500. (D) High magnification of interfascicular microglial cell in the pyramidal tract shows long slender processes which demonstrate presence of the HRP reaction product, Methyl green counterstain, × 800.

procedures resulted in a complete lack of staining (Fig. 2F).

In the normal unoperated facial nucleus very little lectin reactivity was present (Fig. 2A). All motor neurons lacked staining and the only reactive structures were scattered microglia and the endothelium of blood vessels. As early as 24 h after unilateral transection of the facial nerve increased numbers of microglia, relative to the unoperated control side, were noted in the neuropil of the facial nucleus on the operated side. After 48 h this increase was more conspicuous, with many proliferating cells present in the neuropil and some cells occurring perineuronally (Fig. 2B). These actively proliferating microglial cells did not appear hypertrophied compared to microglia in other normal regions of the brain. A maximal microglial response was apparent at days 4 to 6, and at that time most cells were localized in typical perineuronal positions (Fig. 2C, D). During the period of maximal proliferation mitotic figures were easily observed, and could be studied in greater numbers following the administration of colchicine (1 mg ml^{-1} i.p.) 3 to 4 h before sacrifice. Although increased numbers of microglia were already

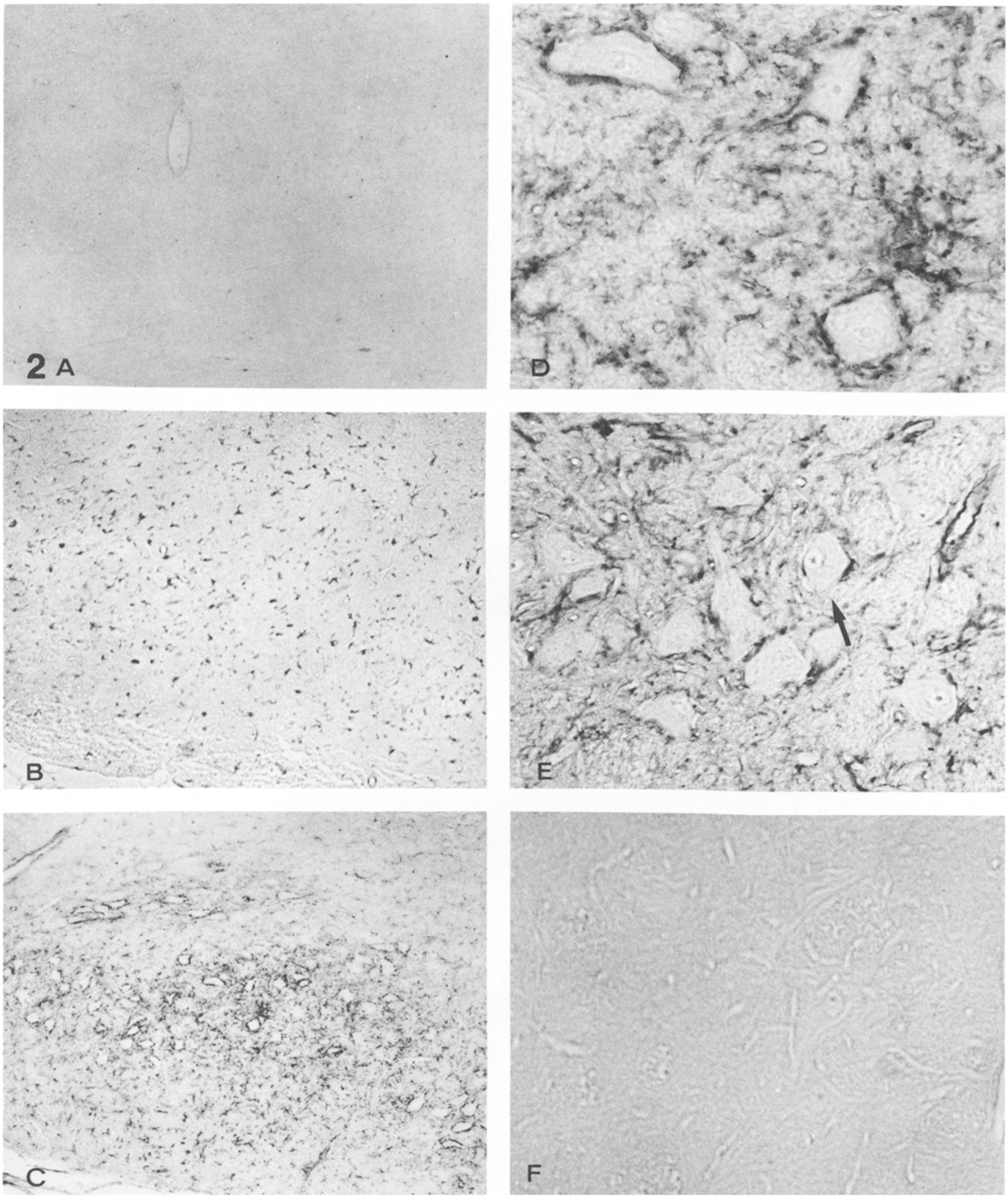


Fig. 2. Demonstration of lectin-reactive microglia during axonal reaction in the facial nucleus. (A) Contralateral unoperated facial nucleus 4 days after axotomy shows very little reactivity. (B) Operated facial nucleus 2 days after nerve section. Note markedly increased numbers of microglia throughout the entire nucleus. (C) Maximal glial proliferative response is seen at 6 days after axotomy. A large proportion of neurons is demarcated clearly by perineuronal glial envelopes. (A–C) × 80. (D) Higher magnification of (C) shows perineuronal infiltrates, × 500. (E) At 4 weeks after axotomy the glial reaction appears attenuated. Neurons are not completely surrounded by glial processes anymore, but chromatolytic features can still be recognized (arrow). (F) Cytochemical control section incubated with α-galactosidase prior to application of GSA I-B_4–HRP lacks staining. (E–F) × 320.

observed 24 h after transection, mitotic figures could not be seen at that time, even after prior injection of colchicine. At 48 h mitotic activity was high as revealed by the presence of numerous mitoses with or without colchicine injections. Cell division was maintained until about day 6, after which no more mitotic figures were seen. While undergoing mitosis the cells became rounded, but retained their lectin affinities. The presence of microglia on the operated side persisted for 3 to 4 weeks after axotomy without apparent loss of cell density; however, after 4 weeks the glial reaction seemed attenuated with fewer cells being present (Fig. 2E). In the contralateral facial nucleus the number of resident microglia did not increase at any time, demonstrating a density of microglia similar to other grey matter regions of the brainstem.

Electron microscopy of lectin binding

Since the unequivocal identification of microglia was difficult to achieve by light microscopy only, an evaluation by ultrastructural cytochemistry was indicated. For this purpose the tissue had to be fixed in aldehydes to obtain good fine-structural preservation, but it also had to be treated with trypsin and incubated with lectin–HRP conjugates in the presence of detergent to facilitate adequate binding. Surprisingly, the preparations showed little tissue and membrane damage as a result of these treatments used prior to and during the staining process. An advantage of enzyme and detergent treatments, also seen light microscopically, was a drastic reduction in non-specific background staining to such an extent that it was virtually absent.

Electron microscopy showed that lectin reactivity was present on the plasma membrane of microglial cells, which were easily distinguished from astrocytes and oligodendrocytes by their size, nuclear morphology, dense cytoplasm and prominent wide cisternae of rough endoplasmic reticulum (Figs 3, 4). Macroglia, in contrast, never showed deposits of reaction product on their plasma membrane or in their cytoplasm (Figs 4, 7). Similarly, motor neurons did not show any staining, except that in sites of close apposition of microglial and neuronal membranes the neuronal plasmalemma appeared stained (Fig. 6). However, this is likely to be due to diffusion of the DAB reaction product. At 4 and 5 days after axotomy, microglia were seen most conspicuously in perineuronal locations sending long cytoplasmic pseudopods to cover large areas of the neuronal soma (Fig. 5). The microglial processes formed multiple convoluted folds, and were seen to surround dendrites, engulfing them almost completely (Fig. 8). Lectin binding sites were also visualized in the microglial cytoplasm. As shown in Fig. 10, the intracytoplasmic structures exhibiting lectin staining appeared to be cisternae of smooth endoplasmic reticulum which were sometimes seen to be in continuity with the rough endoplasmic reticulum. In addition, vesicles in the glial cytoplasm were reactive, as well as other tubular structures possibly representing either smooth endoplasmic reticulum or Golgi cisternae (Fig. 9). Labelled microglial cells were also located near small vessels and capillaries, but they were always situated outside the basal lamina. No labelled pericytes were observed.

Co-localization of microglia and astrocytes

The demonstration of specific lectin affinities for microglia extends ongoing studies in this laboratory concerned with the expression of GFAP during the axonal reaction. The combined use of lectin histochemistry and GFAP immunocytochemistry has allowed simultaneous demonstration of microglia and astrocytes in the same section (Fig. 11).

At 24 h after axotomy, at which time increased numbers of microglia were already apparent, there were no more GFAP-reactive astrocytes in the facial nucleus on the operated side than on the control side. Starting at day 2, when microglia began to show mitotic activity, GFAP-reactive astrocytes became visible on the operated side and increasing numbers of reactive astrocytic profiles appeared until about 3 weeks after axotomy, as described earlier (Graeber & Kreutzberg, 1986). Incidentally, dividing astrocytes were never observed. During the first few weeks microglia accounted for the majority of glial cells present. While during the whole time course of this study, save for the first two days, microglia were for the most part seen in perineuronal locations, cell bodies of reactive astrocytes tended to be situated more in the interneuronal regions; only occasionally were they observed perineuronally, notably during the later time points. At 4 weeks, the latest time interval examined, the glial reaction in the facial nucleus consisted of approximately equal proportions of microglia and astrocytes.

In summary, it can be said that the response of microglia to axotomy is very rapid, peaking during the first week, whereas the astrocytic reaction seems to increase more gradually for several weeks until reaching a maximum around the third or fourth week.

Negative controls for staining of GFAP consisted of omitting the primary antiserum from the staining sequence, and resulted in a complete lack of reactivity. On the other hand, visualization of GFAP-reactive astrocytes in the cerebellum in each section provided a positive control for the specificity of the antibody.

Discussion

It has been demonstrated that resident as well as reactive microglia, which proliferate in response to cranial nerve axotomy, can be selectively stained in the rat with GSA I-B_4–HRP. Since GSA I-B_4–HRP did not show any affinity for astrocytes and oligodendrocytes, lectin binding provides yet another histochemical marker that is useful for studying the response of microglia in various experimentally induced neuropathological conditions.

These findings, on one hand, extend the results of other investigators reporting only staining of blood vessel endothelium with GSA I-B_4–FITC conjugates in rat brain (Peters & Goldstein, 1979), but, on the other hand, directly contradict the findings of Schelper *et al.* (1985) who claimed that proliferating true microglia in the hypoglossal nucleus after axotomy were not labelled with GSA I-B_4 and that lectin binding could be used to distinguish true microglia from monocyte-derived brain macrophages which were said to stain positively with GSA I-B_4. The reason for this contradiction is likely to be found in the source and purity of the lectin or in the different methodology of fixation and incubation parameters employed. Of further interest with regard to the relationship between macrophages and microglia are the findings of Maddox *et al.* (1982), which showed that only stimulated peritoneal macrophages are labelled with GSA I-B_4–FITC whereas resident macrophages are not. Our results show, however, that both resting and reactive microglia are stained, and thus suggest the presence of surface glycoconjugates on microglia which are not altered by external stimuli. One might argue that, stimulated by nerve section, there is an increase in GSA I-B_4 binding sites on resident microglia accounting for higher levels of reactivity seen in the facial nucleus. However, since there is such rapid proliferative activity, as shown in several independent studies (Cammermeyer, 1965; Sjöstrand, 1965; Kreutzberg, 1966), it seems more likely that increased levels of GSA I-B_4 binding are due to an increase in cell number. On the basis of the presented data regarding the origin of microglia, the possibility of an invasion by blood monocytes cannot be excluded, although it should be pointed out that in the present model of facial nerve section, in which surgery occurs outside the CNS, there is no disturbance of the blood–brain barrier. No invasion by blood-borne elements has ever been seen in extensive electron microscopic examination of these tissues. Therefore we are inclined to think that the cells proliferating in the facial nucleus are resident microglia which are stimulated to proliferate by a yet unknown mechanism.

The reaction of GSA I-B_4 is based on its affinity for terminal α-D-galactose residues which are known components of *O*-glycosidic carbohydrate units of rat brain glycoproteins (Finne & Krusius, 1976). In fact, the disaccharide α-galactosyl-(1-3)-*N*-acetylgalactosamine was found by these authors to be brain specific, since it could not be detected in extraneural tissues of the rat. It is possible, therefore, that sites revealing binding of GSA I-B_4 may reflect the localization of this carbohydrate unit.

Alternatively, glycosphingolipids and gangliosides constitute another important group of galactose-containing glycoconjugates which are present in brain in high concentrations (Yu, 1984). Although organic solvents used in histologic processing remove

Fig. 3. The electron micrograph shows a slender microglial cell (mg) situated next to a chromatolytic neuron (N). Lectin reactivity is present all along the microglial cell plasma membrane (arrows). Note the characteristic nucleus with dense heterochromatin and the prominent wide cisternae of rough endoplasmic reticulum. Four days after operation. × 13200.

Fig. 4. The top right of the micrograph shows a microglial cell (mg) adjacent to a dendrite (D) in the centre. Reaction product is present on the plasma membrane surrounding the glial cell body, and a small cytoplasmic process just below the dendrite (arrow). In the lower left another glial cell is shown which exhibits no lectin binding. This is probably an oligodendrocyte (og). × 19400.

Fig. 5. A microglial cell is seen covering a large area of neuronal soma with positively stained cytoplasmic extensions. × 16400.

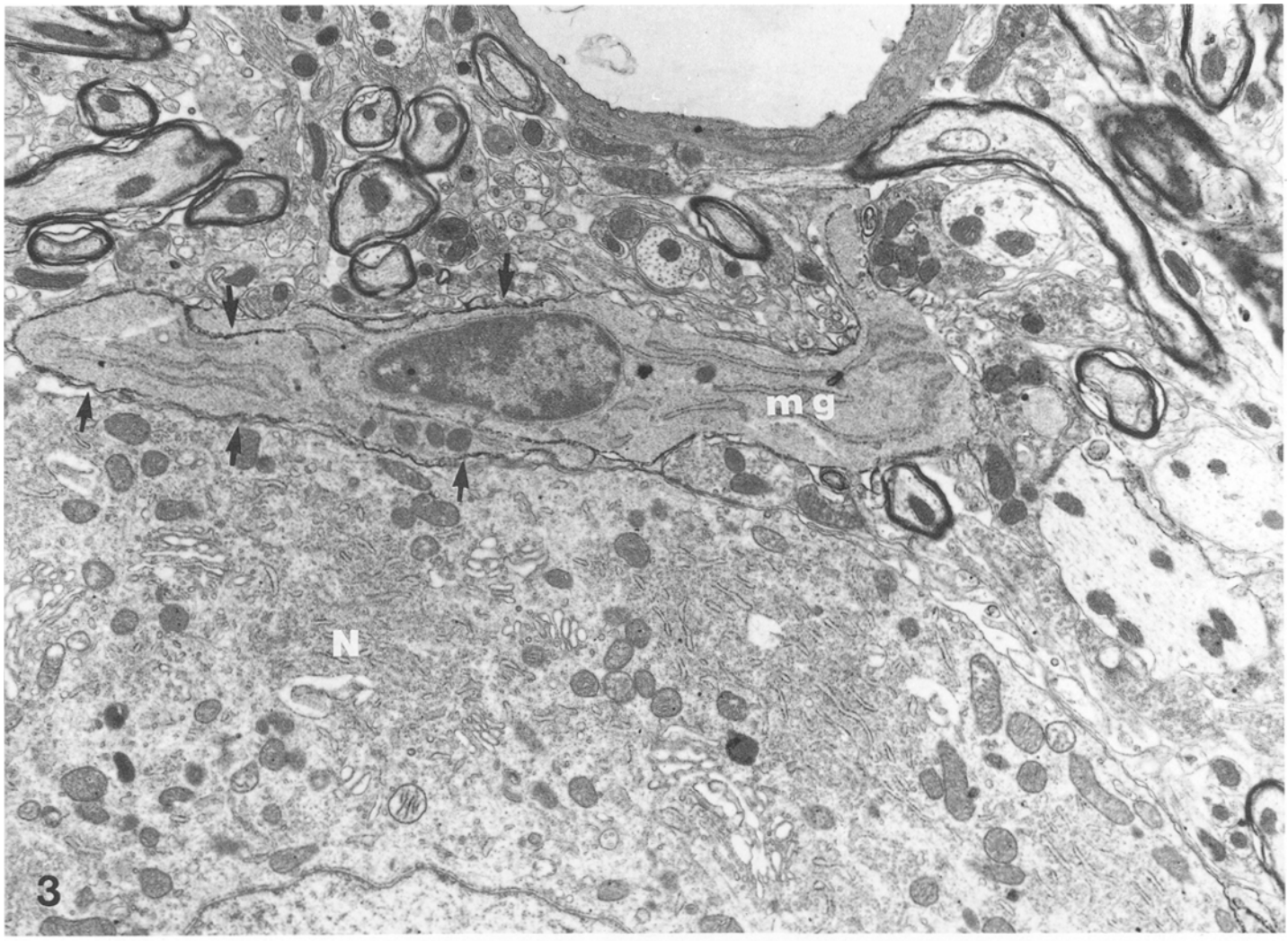
Fig. 6. The glial–neuronal interface shows reaction product present on the neuronal as well as the glial membranes (arrows). × 26700.

Fig. 7. An astrocyte in the regenerating facial nucleus is devoid of reaction product. × 7000.

Fig. 8. A dendrite (D) is shown which is almost completely surrounded by lectin-reactive microglial processes. × 16200.

Fig. 9. Microglial processes demonstrating lectin binding are shown in apposition with a large dendrite (D) in the centre. Reaction product on the membrane is confined to the zone of contact between glia and dendrite, and staining in the cytoplasm is present on structures resembling smooth membrane cisternae (arrows). × 20200.

Fig. 10. Staining in the glial cytoplasm is present in the smooth endoplasmic reticulum (arrow) seen in continuity with the rough endoplasmic reticulum (rer). × 30300.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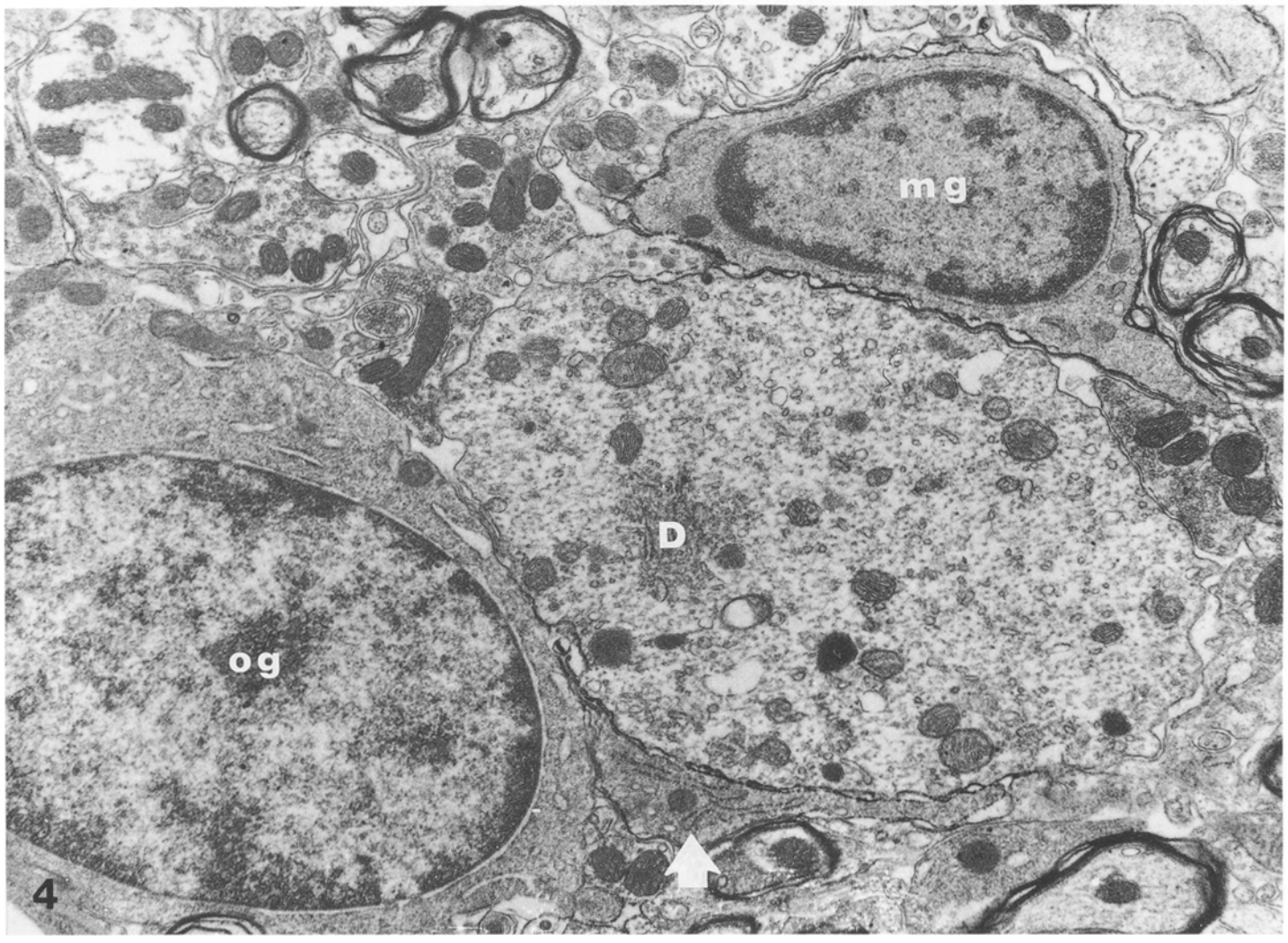
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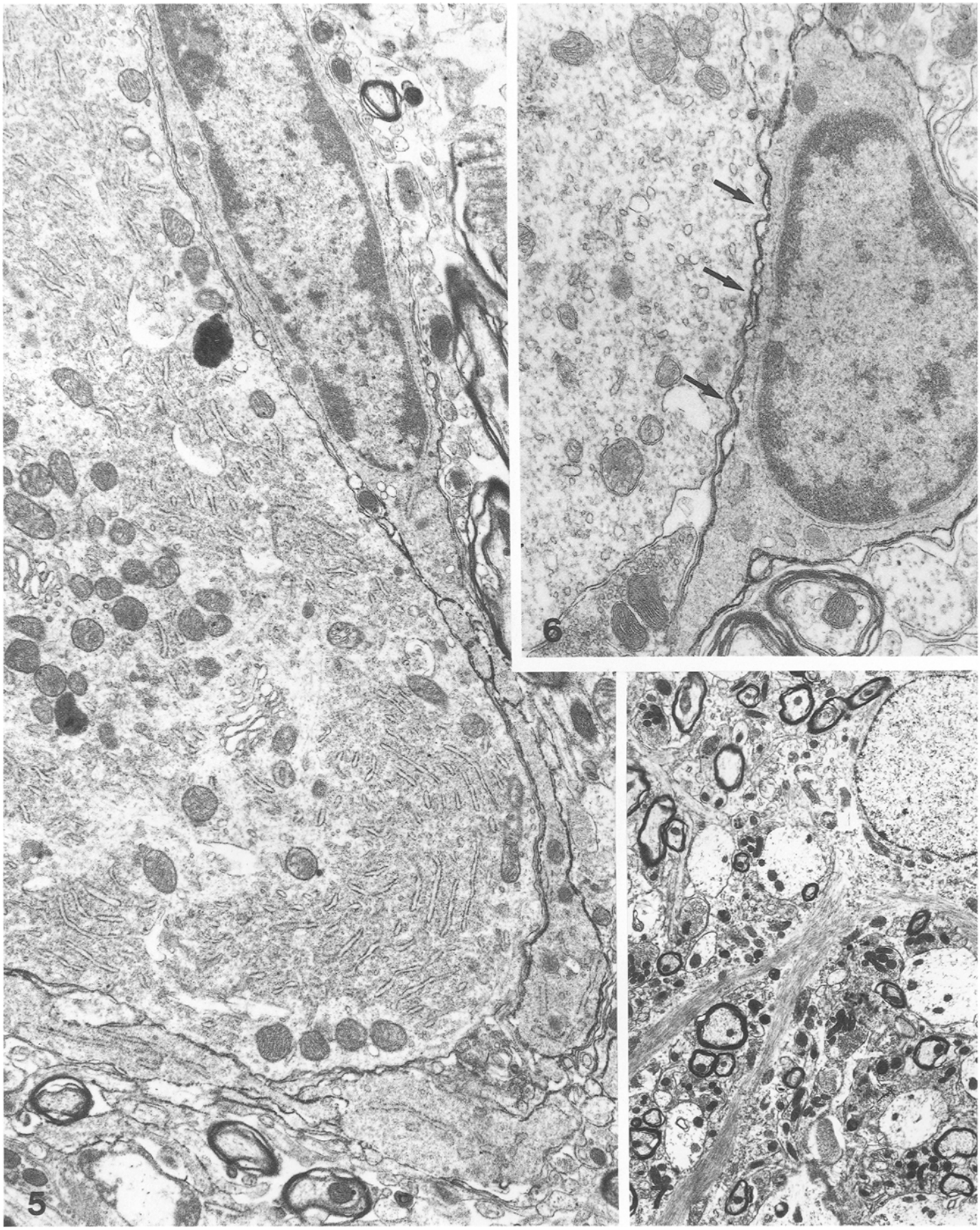
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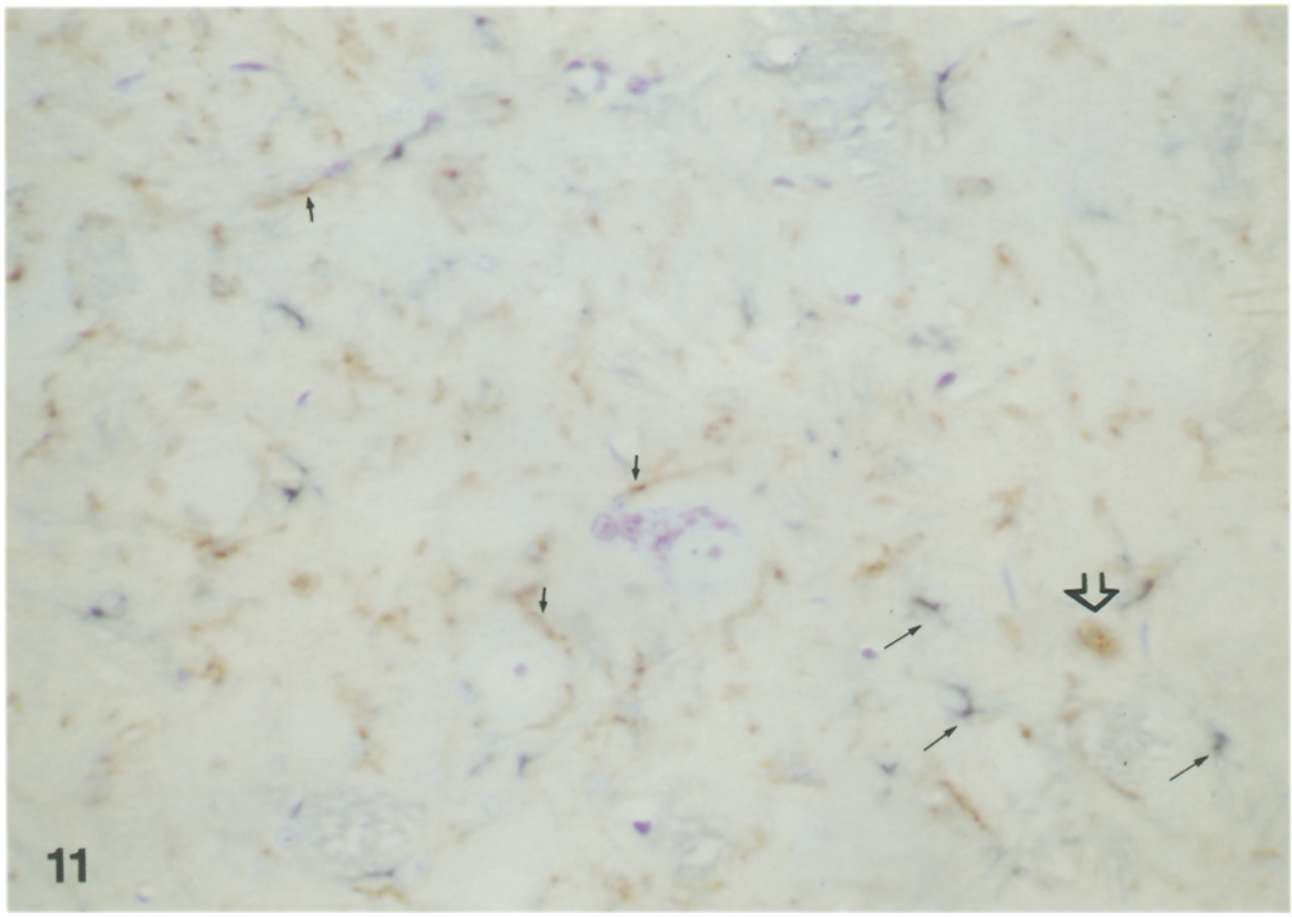
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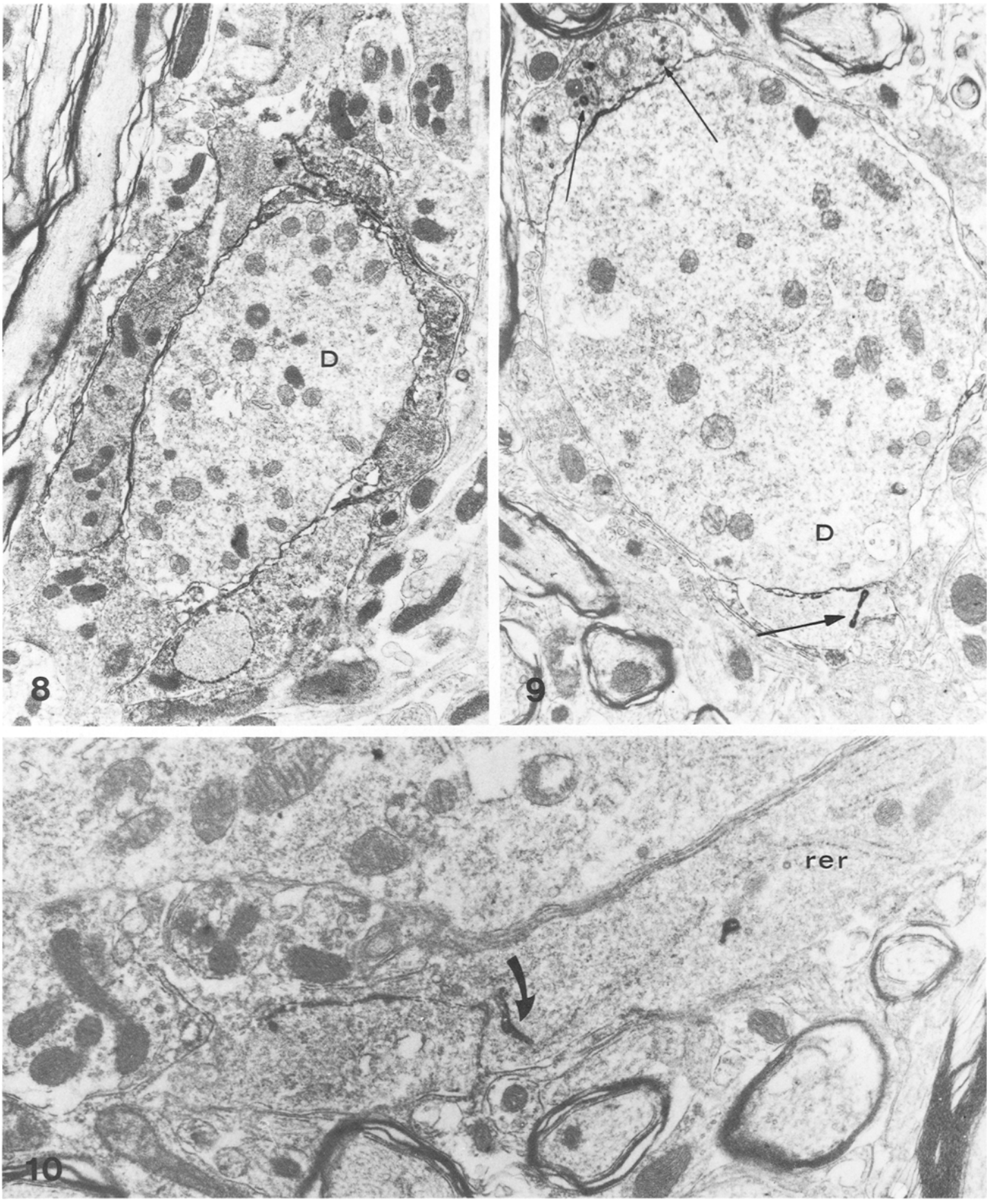

Fig. 11. Co-localization of microglia with GSA I-B_4–HRP (short arrows, brown reaction product), and of astrocytes with a monoclonal antibody to GFAP (long arrows, black reaction product) demonstrates the mixed glial reaction present 3 days after axotomy. At this time the microglial reaction is predominating, and dividing microglial cells become rounded (open arrow). Cresyl echt violet counterstain. × 500.

a large portion of lipidic tissue components, one must consider the possibility that the galactoconjugate bound by GSA I-B_4 may be a glycosphingolipid firmly associated with the microglial plasma membrane and thus resistant to extraction by xylene or ethanol. The glycoconjugate probably exists in a non-sialylated form, as α-linked galactose residues are not thought to constitute receptors for sialic acid, and known α-galactose-containing glycolipids do not contain neuraminic acid (Finne & Krusius, 1976). Hence, the microglial glycoconjugate would, by definition, be considered a neutral glycosphingolipid and not a ganglioside. Studies aimed at determining the nature of this substance, as well as the structure of its penultimate sugar sequences, are presently being conducted in this laboratory.

The histochemical analysis of brain tissue with lectins has revealed specific localizations of glycoconjugates in various brain regions (Hatten *et al.*, 1979; Brückner *et al.*, 1985; Nakagawa *et al.*, 1986). In view of physiologic studies demonstrating that sugar residues are essential for assuring proper functional activity of complex carbohydrates (Langer *et al.*, 1976; Sattler *et al.*, 1977), the histochemical visualization of these substances has served to correlate the occurrence of a glycoconjugate in a given site with a potential physiologic function. This has led to claims that brain glycoconjugates play important functional roles in such diverse processes as myelination (Wood & McLaughlin, 1975; Sternberger *et al.*, 1979), receptor-mediated interactions (Nakagawa *et al.*, 1986), morphogenesis (Brückner *et al.*, 1985) and learning and memory (Pohle *et al.*, 1982). In previous publications (Streit *et al.*, 1985b, 1986) it was shown that binding of GSA I-B_4–HRP in the CNS is not limited to microglia, but that this lectin also shows a strong affinity for unmyelinated primary afferent fibres and terminals in the substantia gelatinosa of the spinal cord and brainstem, yielding a staining pattern congruent with substance P immunoreactivity. The overlap between lectin reactivity and the content of substance P was taken to suggest a possible role of galactose-containing glycoconjugate in mechanisms of nociception. With regard to microglial staining, however, this suggestion does not seem very plausible, and brings up the concept of microheterogeneity of brain glycosubstances. The fact that GSA I-B_4 recognizes the presence of the same terminal sugar in functionally quite unrelated CNS structures, i.e. microglia and sensory fibres, does not necessarily imply that these elements share identical glycoconjugates. On the contrary, it favours the idea of variation being present in the penultimate and neighbouring sugar sequences, as well as in the branching patterns of the individual oligosaccharides. This microheterogeneity, in turn, is possibly a reflection on the different functional capacities of galactoconjugates in different sites. A detailed *in situ* sequencing analysis of oligosaccharides using successive exoglycosidase treatments is therefore necessary to unravel these oligosaccharide structures. At this time a definite physiologic function can not be ascribed to the microglial glycosubstance, but several possibilities exist. In the context of the present communication it is relevant to speculate that the galactoconjugate on the microglial plasma membrane has a function related to the proliferative response of microglia during the retrograde reaction. One could imagine it to be a kind of adhesion molecule which mediates and maintains the close apposition of microglial and neuronal membranes during the period of regeneration. Alternatively, several reports ascribe receptor functions to brain glycoproteins (Gioannini *et al.*, 1982; Shirakawa *et al.*, 1983) and glycolipids (Rosenberg, 1979), and one might think of the microglial glycoconjugate as a receptor which possibly binds to a growth factor released by facial motor neurons in response to axotomy. Conceivably, this hypothetical interaction of a glial receptor with a neuronally released substance could form the signal for the subsequent microglial proliferation. In this regard it is of interest that microglia themselves have been shown to release so-called 'glia promoting factors' which are responsible for the regulation of astrological proliferation (Giulian & Baker, 1985). This observation is compatible with our findings, obtained by double labelling, that microglial proliferation occurs as early as 24 h after axotomy, and that GFAP-reactive astrocytes are seen only after the microglial reaction has already been initiated.

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