

# Molecular Specialization of Astrocyte Processes at Nodes of Ranvier in Rat Optic Nerve

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**Abstract.** The HNK-1 and L2 monoclonal antibodies are thought to recognize identical or closely associated carbohydrate epitopes on a family of neural plasma membrane glycoproteins, including myelin-associated glycoprotein, the neural cell adhesion molecule, and the L1 and J1 glycoproteins, all of which have been postulated to play a part in mediating cell-cell interactions in the nervous system. We have used these two antibodies in immunofluorescence and immunogold-electron microscopic studies of semithin and ultrathin frozen sections of adult rat optic nerve, respectively, and we show that they bind mainly to astrocyte processes around nodes of Ranvier. Most other elements of the nerve, including astrocyte cell bodies and large

astrocytic processes, are not labeled by the antibodies. To our knowledge, this is the first demonstration that perinodal astrocyte processes are biochemically specialized. We provide evidence that one of the HNK-1<sup>+</sup>/L2<sup>+</sup> molecules concentrated around perinodal astrocyte processes is the J1 glycoprotein; our findings, taken together with previously reported observations, suggest that the other known HNK-1<sup>+</sup>/L2<sup>+</sup> molecules are not concentrated on these processes. Since anti-J1 antibodies previously have been shown to inhibit neuron to astrocyte adhesion in vitro, we hypothesize that J1 may play an important part in the axon-glial interactions that presumably are involved in the assembly and/or maintenance of nodes of Ranvier.

**I**N the mammalian central nervous system, the myelin sheath is composed of repeated segments formed by individual oligodendrocyte processes. The gaps between these segments constitute the nodes of Ranvier, which in the adult rat optic nerve average 0.8–0.9  $\mu\text{m}$  long (11). The axon plasma membrane at the nodes is different from that in the myelinated internodal segments: for example, the voltage-gated Na<sup>+</sup> channels, which are responsible for propagating action potentials, are thought to be highly concentrated at the node (32, 41), as has been demonstrated in the peripheral nervous system (8, 31). By largely confining electrical excitability to nodes, myelinated axons maximize the velocity and efficiency with which they conduct action potentials (12). In the optic nerve (11, 40) and spinal cord (9, 29), the exposed axon at the nodes is partially enclosed by astrocyte processes. The assembly and maintenance of the nodal and internodal specializations probably involve complex interactions between neurons and both oligodendrocytes and astrocytes; those between astrocytes and axons may be as important as those between oligodendrocytes and axons, since node-like specializations in structures involving only axons and astrocyte-like Müller cells have been seen in the oligodendrocyte-free nerve fiber layer of the rat retina (3, 10). The molecular mechanisms that mediate these interactions between glial cells and axons are unknown.

In the experiments reported here, we have used immuno-

fluorescence and immunogold labeling of semithin and ultrathin frozen sections, respectively, to show that astrocyte processes around nodes of Ranvier in adult rat optic nerve are antigenically distinct: they are preferentially labeled by the monoclonal antibodies HNK-1 (1) and L2 (17). The HNK-1 antibody (also called anti-leu-7) was originally raised against a T cell line and shown to recognize human natural killer cells (1); recently it has been shown to bind to a variety of neural tissues, including human central and peripheral myelin (34) and developing rodent oligodendrocytes, astrocytes, and neurons (23, 42). The L2 antibody was raised against a membrane fraction of mouse cerebellum, and recognizes an antigenic determinant identical to, or closely associated and simultaneously expressed with, the HNK-1 determinant; the use of either antibody blocks immunolabeling with the other (17). This determinant has been reported to be a carbohydrate (17, 28) that is present on a family of neural plasma membrane glycoproteins, including myelin-associated glycoprotein (MAG)<sup>1</sup> (22), the neural cell adhesion molecule (N-CAM), and the L1 (17) and J1 glycoproteins (16), all of which have

<sup>1</sup> Abbreviations used in this paper: G anti-MIg-Rh, goat anti-mouse immunoglobulin conjugated to rhodamine; GFAP, glial fibrillary acidic protein; MAG, myelin-associated glycoprotein; N-CAM, neural cell adhesion molecule; NF, neurofilament protein; PB, phosphate buffer, pH 7.4; PBLs, PB containing 20 mM lysine and 0.02% saponin; Sh anti-R1g-F1, sheep anti-rabbit immunoglobulin conjugated to fluorescein.

been postulated to play a part in mediating cell-cell interactions in the nervous system (4, 16, 17, 21, 28, 33). We provide immunocytochemical and immunoblotting evidence that at least one of the molecules recognized by the HNK-1 and L2 antibodies on perinodal astrocyte processes is the J1 glycoprotein, whereas our findings, together with previously reported observations (30, 36), suggest that MAG, N-CAM, and L1 are not concentrated on these processes. Since anti-J1 antibodies have been shown to bind to the surface of glial cells and to inhibit neuron-astrocyte adherence *in vitro* (16), our findings suggest that J1 may play a role in the cell-cell interactions involved in the assembly and/or maintenance of the node of Ranvier.

## Materials and Methods

### Frozen Sections

Optic nerves from adult (>3 mo) Sprague-Dawley rats were prepared as previously described (24, 25) with some modification. The rats were perfused through the ascending aorta with a solution of 3% paraformaldehyde (Fluka AG, Buchs, Switzerland), 0.5% glutaraldehyde (Emscope Laboratories Ltd, Ashford, Kent, England) and 7% sucrose in phosphate buffer, pH 7.4 (PB). The optic nerves were removed, cut into short lengths, and postfixed in the same fixative for 1 h. They were then transferred to a 2 M solution of sucrose in PB for an hour more before freezing onto a copper stub with a slush of Freon 22 cooled in a bath of liquid nitrogen. In experiments using anti-N-CAM and anti-L1 antisera, optic nerves were fixed in 4% paraformaldehyde without glutaraldehyde.

For immunofluorescence, 0.5- $\mu$ m semithin sections were cut on a dry glass knife at  $-65^{\circ}\text{C}$  using a Sorvall MT2B microtome with FTS/LTC-2 frozen thin sectioner cryoattachment (Sorvall Instruments Div., E. I. Du Pont de Nemours & Co., Inc., Newtown, CT), then transferred to coverslips with a drop of saturated sucrose (37). For immunoelectron microscopy, ultrathin sections of  $\sim 100$  nm (giving a purple-gold interference pattern) were cut at  $-95^{\circ}\text{C}$ , and transferred by the same method to Formvar-carbon coated nickel grids, which had been briefly ionized in a glow discharge unit.

### Immunofluorescence Staining of Semithin Frozen Sections

Before staining, all sections were washed in PB containing 20 mM lysine and 0.02% saponin (Sigma Chemical Co., Poole, Dorset, England) (PBLs), and this solution was used throughout the staining procedure. The sections were then placed in 50% normal goat serum in 2.5% bovine serum albumin (BSA, Sigma fraction V) for 10 min to block nonspecific antibody binding. They were then sequentially immersed in the specified antibodies followed by the appropriate fluorescent conjugates, each diluted in 5% BSA and incubated for 30 min at room temperature, with 5–10-min washes in PBLs between incubations.

All the antibodies used have been previously described. Two monoclonal antibodies were used: HNK-1 (an IgM [1]) was used as culture supernatant diluted 1 in 2, and L2 (an IgG1 [17]) was used as ascites fluid diluted 1 in 100. Five rabbit antisera were used: anti-gial fibrillary acidic protein (GFAP [27]) diluted 1 in 1,000; anti-J1 (16) diluted 1:400; and anti-155-kD neurofilament protein (NF [2]), anti-N-CAM (26), and anti-L1 (30), all diluted 1 in 50. Both the mouse HNK-1 and rat L2 monoclonal antibodies were detected with goat anti-mouse immunoglobulin conjugated to rhodamine (G anti-MIg-Rh, Cappel Laboratories, West Chester, PA), and the rabbit antibodies were detected with sheep anti-rabbit immunoglobulin conjugated to fluorescein (Sh anti-RIg-F1, Wellcome Diagnostics, Dartford, England), both diluted 1 in 100. In double-labeling experiments with monoclonal and rabbit antibodies, unless stated otherwise, the sequence of incubations was monoclonal antibody, G anti-MIg-Rh, rabbit antisera, Sh anti-RIg-F1. Control experiments showed that the Sh anti-RIg-F1 and G anti-MIg-Rh did not cross-react with the monoclonal antibodies or rabbit antisera, respectively. After staining, the coverslips were mounted in phosphate-buffered saline containing 2.5% 1,4 diazo bicyclo(2,2,2)-octane (BDH Chemicals Ltd, Dagenham, Essex, England) to inhibit fading of the fluorescence (13), examined under a Zeiss Universal incidence fluorescence microscope equipped with phase contrast, fluorescein, and rhodamine optics, and photographed on Tri-X film rated 400 ASA.

In control experiments, no significant staining was seen with the fluorescent conjugates alone, or when ascites fluid obtained from mice carrying a non-Ig-secreting myeloma tumor or normal rabbit serum (diluted 1 in 100) was used

instead of the monoclonal or rabbit antibodies, respectively. Moreover, no significant labeling was seen with supernatant containing an IgM monoclonal antibody, 38D7, which reacts specifically with neurons of the peripheral nervous system (39).

### Immunogold Staining of Ultrathin Frozen Sections

The method used was a modification of that described by Griffiths (6) and Slot and Geuze (35). Ultrathin sections on grids were washed on droplets of PBLs and passed sequentially through droplets of solution on parafilm. After 10 min in 50% normal goat serum in 2.5% BSA diluted in PBLs they were exposed to antibody for 30 min, then washed with three changes of PBLs for 5 min each. The HNK-1 and L2 antibodies were used at the same dilution as for immunofluorescence; in addition, RT97 (an antibody against the 210-kD NF [43]) was used as ascites fluid diluted 1 in 100. Two methods of immunogold labeling were then employed. The L2 and RT97 antibodies were detected with a G anti-MIg antibody coupled to 10-nm colloidal gold particles (G anti-MIg G10, Janssen Pharmaceutica, Beerse, Belgium) incubated for 30 min at a dilution of 1 in 5, whereas the HNK-1 was detected using an amplifying intermediate layer of a  $\text{F(ab')}_2$  fraction of a rabbit anti-MIg prepared as previously described (7) and used at a 1 in 100 dilution in PBLs containing 5% BSA, followed by a goat anti-rabbit immunoglobulin coupled to 10-nm colloidal gold (G anti-RIg-G10, Janssen) diluted 1 in 10. All gold labeling was performed in 20 mM Tris/saline, pH 8.2, containing 1% BSA, and grids were washed before and after this stage with Tris/saline containing 0.1% BSA. After immunolabeling, grids were further fixed in 0.5–1.0% glutaraldehyde in PB for 10 min before embedding.

Two different embedding techniques were employed, one using methylcellulose (6, 38) and the other using Epon (15). In the former case, grids were stained with 0.5% aqueous uranyl acetate for 5 min and then transferred via distilled water to three drops of 1.5% methylcellulose (Fluka AG) on ice for a total of 1 min. Grids were picked up in a 4-mm loop, the excess methylcellulose removed and the grids were allowed to dry. For Epon embedding, grids were osmicated with 1% osmium tetroxide in 0.05 M PB for 10 min, washed in 7% sucrose in distilled water, and then stained with 0.4% aqueous uranyl acetate in 5% sucrose. They were then dehydrated in ethanol, infused with 2% Epon 812 (Emscope Laboratories Ltd) in absolute alcohol and allowed to dry before polymerization at  $60^{\circ}\text{C}$  overnight. The Epon-embedded grids were also post-stained with 0.04% alkaline bismuth subnitrate (Mallinckrodt Inc, St Louis, MO 63134). Grids were examined in a JEOL 100CX electron microscope at either 80 or 100 kV.

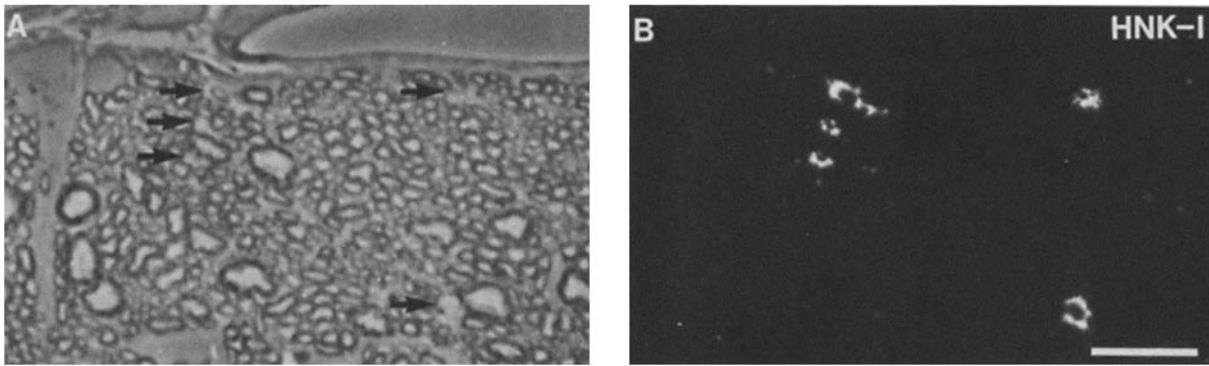
### Conventional Electron Microscopy

Adult rats were perfused with the same fixative used for the preparation of frozen sections, and then the optic nerves were removed and postfixed overnight at  $4^{\circ}\text{C}$ . They were then washed in PB, placed in 1% osmium tetroxide in PB for 2 h at  $4^{\circ}\text{C}$ , washed in distilled water, and stained en bloc with 0.5% aqueous uranyl acetate for a further 2 h, after which they were dehydrated in graded alcohols and embedded in Epon 812. Thin sections were poststained with uranyl acetate and lead citrate.

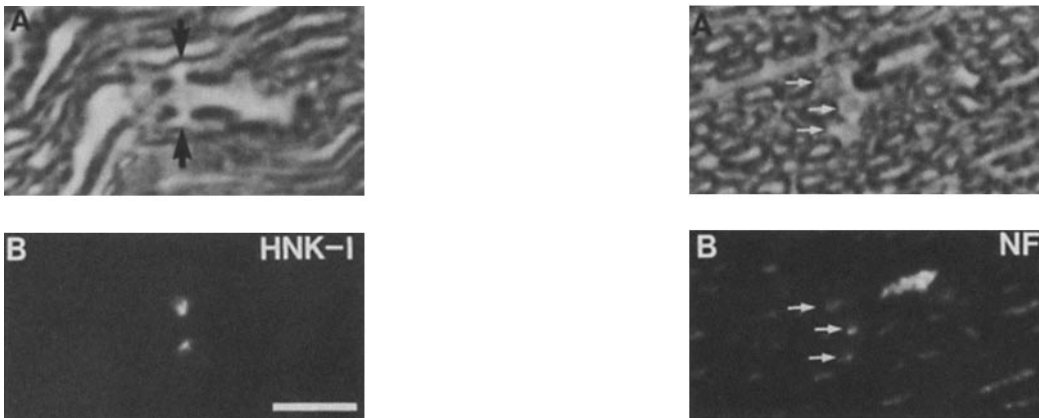
### Immunoblotting

Adult rats were killed by cervical dislocation and their optic nerves were removed. The nerves were placed immediately into PBS containing 5 mM EDTA and 0.25 mM phenylmethylsulfonyl fluoride at  $0^{\circ}\text{C}$  and then crushed between glass slides and boiled in 0.1 M Tris buffer, pH 6.8, containing 2% SDS and 10% 2-mercaptoethanol for 3 min; the sample was then frozen at  $-20^{\circ}\text{C}$  until it was analyzed.

SDS PAGE, protein transfer to nitrocellulose, and immunolabeling were performed as described by Lawson (20). In brief, samples were run on 5–15% polyacrylamide gradient gels, with approximately one pair of nerves per slot, adjacent to dye-coupled molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD), transferred to nitrocellulose, and incubated in 5% hemoglobin (Hb) overnight. For immunautoradiography, the L2 mouse ascites fluid was used at 1 in 200, whereas the rabbit antisera were used at the following concentrations: anti-N-CAM at 1 in 200, anti-L1 at 1 in 150, and anti-J1 at 1 in 1,000. After 2 h of incubation in the relevant antibody diluted in 1% Hb in PBS, the nitrocellulose was washed in 1% Hb in PBS for 30 min and incubated for 2 h more in the same medium containing an  $\text{F(ab')}_2$  preparation of either rabbit anti-mouse IgG or affinity-purified goat anti-rabbit IgG (a kind gift of Dr. D. Lawson) coupled to  $^{125}\text{I}$  prepared as previously described (20) and used at  $250 \times 10^3$  cpm/ml. After washing, the nitrocellulose was dried and autoradiographed. The exposure time required for the monoclonal antibody (4 wk) was considerably greater than that needed for the antisera (24 h).



**Figure 1.** Transverse semithin frozen section of adult rat optic nerve labeled with HNK-1 antibody as described in Materials and Methods. (A) Phase contrast; (B) rhodamine labeling with HNK-1 antibody. Note the intense labeling of small, phase-lucent, ring-like areas located between the myelinated axons, and the much weaker, punctate staining scattered through the nerve. The pial surface is at the top of the micrographs. Bar, 10  $\mu\text{m}$ .

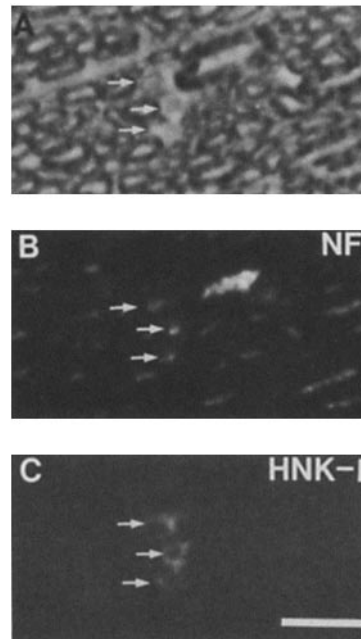


**Figure 2.** Longitudinal semithin frozen section of optic nerve labeled and photographed as in Fig. 1. A node of Ranvier can be seen by phase contrast (arrows in A). Note that only the perinodal area is HNK-1<sup>+</sup> (B); no labeling is present over the compact myelin or axon. Bar, 5  $\mu\text{m}$ .

## Results

### Immunofluorescence

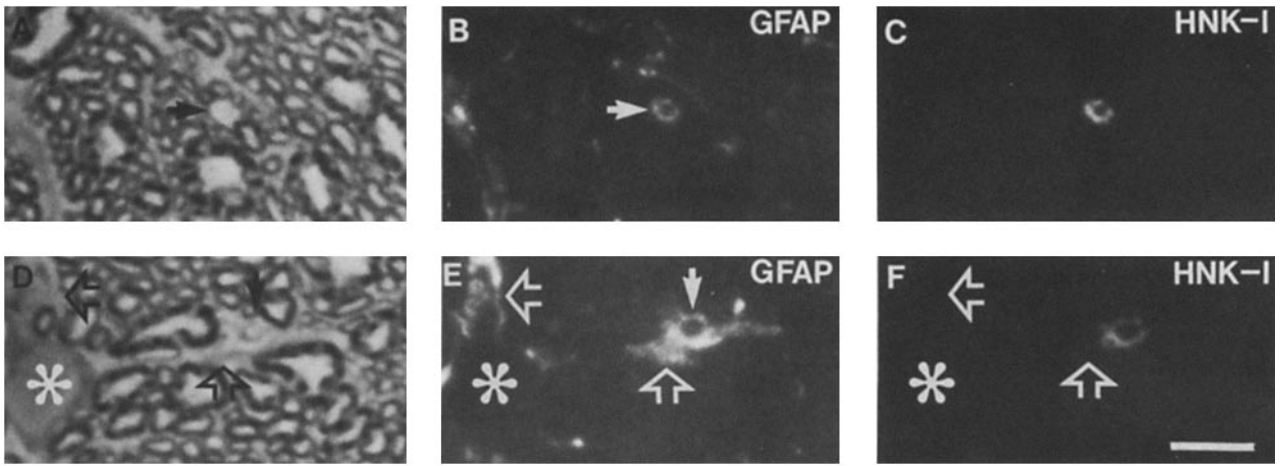
In transverse, semithin frozen sections of adult rat optic nerve, HNK-1 antibody brightly labeled small, phase-lucent areas throughout the nerve in a complete or incomplete ring pattern (Fig. 1). In longitudinal sections these labeled areas were seen to be associated with nodes of Ranvier (Fig. 2). When transverse sections were double-labeled with HNK-1 antibody and with rabbit anti-GFAP or anti-NF antiserum, these HNK-1<sup>+</sup> areas were invariably NF<sup>-</sup> (Fig. 3) and GFAP<sup>+</sup> (Fig. 4), but they always surrounded an NF<sup>+</sup> axon (Fig. 3). In addition when nodes were identified by the characteristic ring-like labeling of phase-lucent areas with anti-GFAP antiserum, these GFAP<sup>+</sup> areas were always HNK-1<sup>+</sup>. Thus the HNK-1 staining of the nodes seemed to be associated with small, perinodal astrocyte processes. Astrocyte cell bodies and large processes were, however, always HNK-1<sup>-</sup>, even when immediately next to a node (Fig. 4). On the other hand, in longitudinal sections, some fine, longitudinally oriented processes were weakly HNK-1<sup>+</sup> producing a speckled pattern of weak staining between axons in transverse sections (which is difficult to see in Fig. 1).



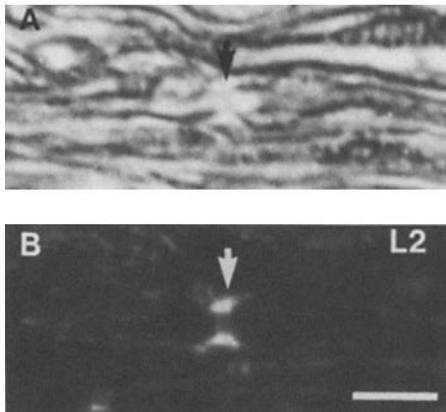
**Figure 3.** Transverse semithin frozen section of adult optic nerve, double-labeled with HNK-1 and anti-NF antibodies, as described in Materials and Methods. (A) Phase contrast; (B) fluorescein labeling with anti-NF serum; (C) rhodamine labeling with HNK-1 antibody. Note that at each of the three nodes of Ranvier (arrows in A), the HNK-1<sup>+</sup>, NF<sup>-</sup> rings (C) surround NF<sup>+</sup>, HNK-1<sup>-</sup> axons (B). Bar, 5  $\mu\text{m}$ .

The same pattern of staining was seen with L2 antibody, although the fluorescence was of greater intensity so that the fine longitudinal processes were more clearly labeled in longitudinal sections (Fig. 5), and the speckled pattern seen in transverse sections was enhanced (Fig. 6C). Some, but not all, of these processes were GFAP<sup>+</sup> in double-labeling experiments. The staining of these longitudinal processes by L2 and HNK-1 antibodies was always less intense than that at the node. In those cases in which stained processes could be seen leading into a node, the staining increased greatly in the region of the node itself (Fig. 5).

HNK-1 and L2 antibodies have been shown to recognize a carbohydrate epitope shared by several different neural glycoproteins, including MAG, N-CAM, and the L1 and J1



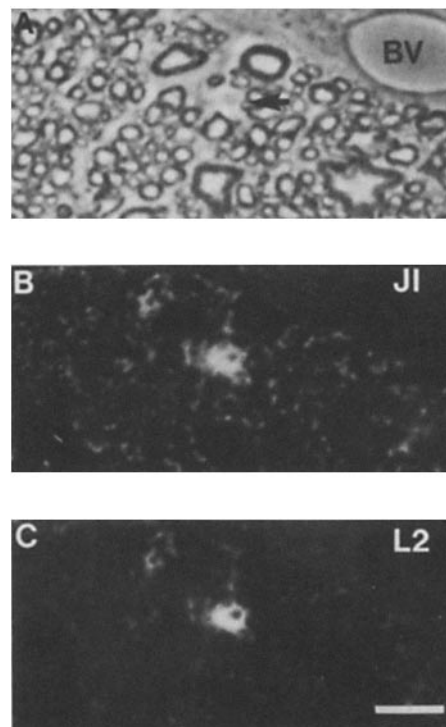
**Figure 4.** Two different transverse semithin frozen sections of optic nerve, double-labeled with HNK-1 and anti-GFAP antibodies. (A and D) Phase contrast; (B and E) fluorescein labeling with anti-GFAP serum; (C and F) rhodamine labeling with HNK-1 antibody. A single node (solid arrows in A and D) is seen in each section. The HNK-1<sup>+</sup> perinodal areas (C and F) are GFAP<sup>+</sup> (B and E). Note that the GFAP<sup>+</sup> astrocyte cell body (asterisk in D-F) and large, GFAP<sup>+</sup>, astrocyte processes (open arrows in D-F) are HNK-1<sup>-</sup> (F), even though one process is adjacent to the node. Bar, 5  $\mu$ m.



**Figure 5.** Longitudinal semithin frozen section of optic nerve labeled with L2 antibody. (A) Phase contrast; (B) fluorescein labeling with L2 antibody. Note that the perinodal area (arrow) is intensely L2<sup>+</sup> whereas there is weaker staining of a number of longitudinally orientated processes, some of which extend to the node. Bar, 5  $\mu$ m.

glycoproteins (16, 17). We found that treating semithin frozen sections with sodium periodate (0.07 M at pH 4.5 for 20 h in the dark at 37°C (as in ref 17) greatly reduced the staining with HNK-1 and L2 antibodies, consistent with the view that the perinodal epitope recognized by these antibodies was a carbohydrate.

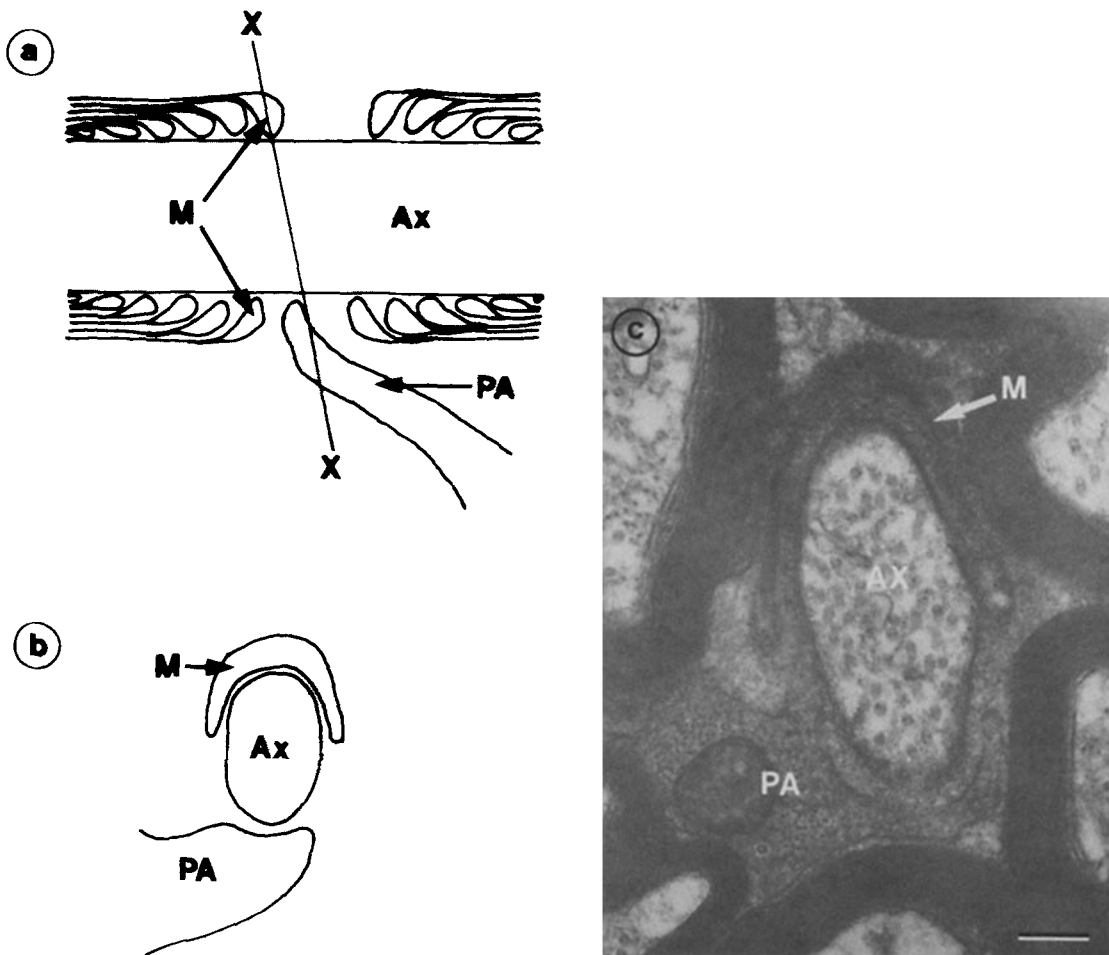
To determine whether the perinodal labeling seen with these antibodies was due to the presence of N-CAM, L1, or J1 glycoproteins, rabbit antisera against these glycoproteins were used before HNK-1 antibody in double-labeling experiments. The J1 antiserum labeled optic nerve in the same pattern as, but more intensely than, the HNK-1 and L2 antibodies, with bright labeling of perinodal areas and a somewhat lower level of labeling over fine longitudinal processes (Fig. 6). As with the monoclonal antibodies, there was no labeling of astrocyte cell bodies or large processes (Fig. 6). In contrast, HNK-1<sup>+</sup>/L2<sup>+</sup> perinodal areas were not specifically labeled by antisera to L1 or N-CAM, even through the latter antiserum brightly labeled the large transverse astrocytic processes (not shown).



**Figure 6.** Transverse semithin frozen section of optic nerve double-labeled with L2 and J1 antibodies as described in Materials and Methods. (A) Phase contrast; (B) fluorescein labeling with anti-J1 serum; (C) rhodamine labeling with L2 antibody. The pattern of labeling with both antibodies is the same, with intense staining around the node (arrow in A) and weaker labeling of longitudinally orientated fine processes. Note that the large astrocyte process on the left of the blood vessel (BV in A) is unlabeled by either antibody. Bar, 5  $\mu$ m.

### Immunogold Electron Microscopy

Although double labeling experiments with HNK-1 and anti-GFAP antibodies suggested that the HNK-1 staining was associated with astrocyte processes in the region of the node of Ranvier, it was not possible to establish unambiguously the cellular location of the antigen by light microscopy. For



**Figure 7.** An illustration of how nodes of Ranvier with perinodal astrocyte processes were identified in transverse ultrathin sections. By selection of slightly oblique sections, some of the nodes can be recognized by the presence of paranodal oligodendrocyte loops on one side of the axon and perinodal astrocyte processes on the other. This is shown schematically in *a* and *b*: the oblique plane of section [*X-X* in *a*] will give the appearance shown in *b*. A conventional electron micrograph of such an oblique section is shown in *c*. *Ax*, nodal axon; *PA*, perinodal astrocyte process; *M*, oligodendrocyte paranodal loop. Bar, 0.2  $\mu\text{m}$ .

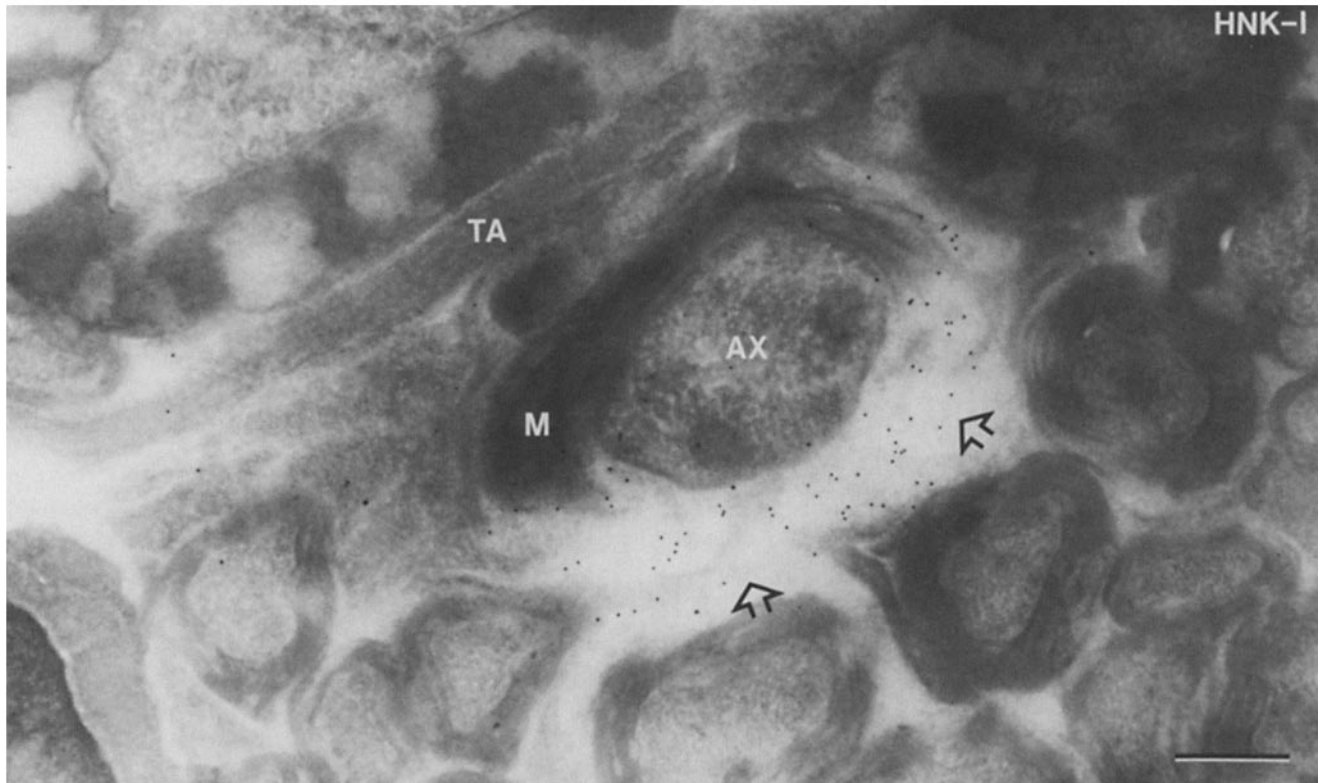
this reason, ultrathin transverse frozen sections of optic nerves were labeled with HNK-1 and L2 antibodies using gold-coupled anti-Ig reagents to visualize the monoclonal antibodies in the electron microscope. Nodes of Ranvier were recognized in slightly oblique transverse sections by their characteristic morphology, as illustrated in Fig. 7. Although occasional gold particles were seen over axons or myelin, the only structures that were consistently labeled above background levels by either of these antibodies were the perinodal areas (Fig. 8). Whereas it was difficult to be certain in nonosmicated sections embedded in methylcellulose what elements in the perinodal regions were labeled (Fig. 8), with the improved morphology of osmicated sections embedded in Epon the labeling was seen to be almost entirely associated with the small astrocyte processes surrounding the nodal axolemma (Fig. 9). In all such nodes examined the level of labeling of these processes was 4–20 times greater than the background labeling in the same section. Whereas most of the labeling around the nodes was associated with the surface or cytoplasm of the perinodal astrocyte process, sections were seen in which some of the labeling appeared to be in the adjacent extracellular matrix. Although a low level of labeling often was seen over the oligodendrocyte paranodal loops, there was little, if

any, specific labeling of nodal axolemma or of astrocyte processes and myelin outside nodal areas (Fig. 8).

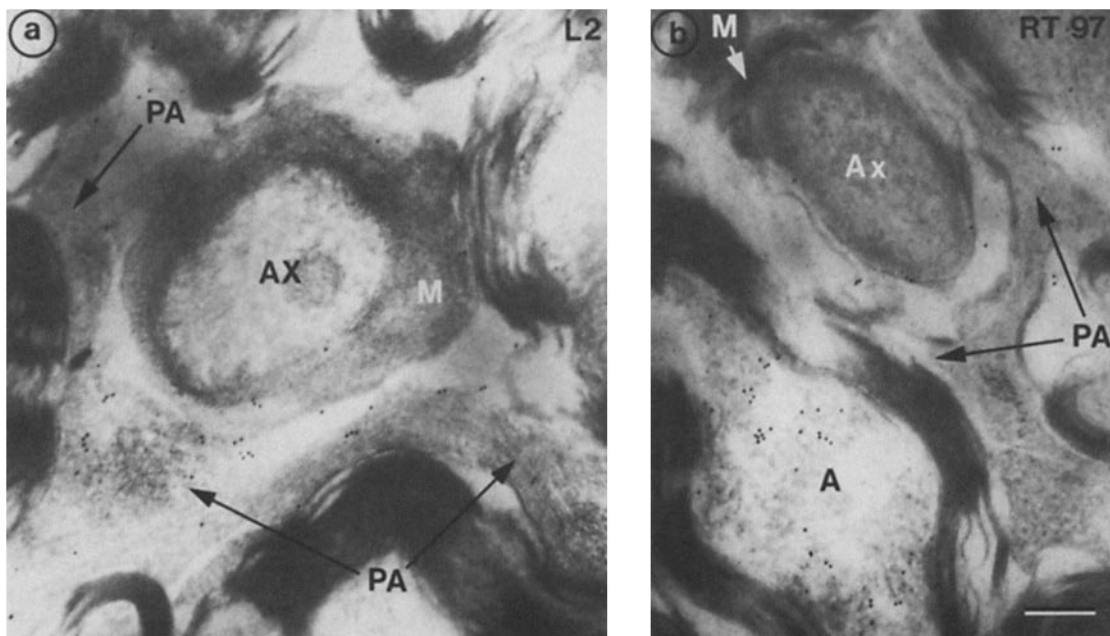
Control experiments showed that there was no significant labeling if the HNK-1 (IgM) or L2 (IgG1) antibodies were omitted or replaced by the 38D7 (IgM) antibody, which reacts specifically with neurons of the peripheral nervous system (not shown). Moreover, when the RT97 (IgG1) anti-NF antibody was used, large axons were labeled intracellularly, whereas the perinodal astrocyte processes were unlabeled (Fig. 9).

#### **Immunoblotting**

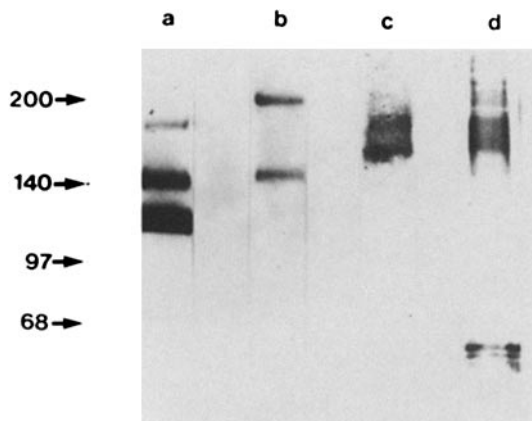
To determine the nature of the molecules expressing the HNK-1/L2 determinant in adult optic nerve, we performed immunoblotting experiments with optic nerve proteins blotted onto nitrocellulose after SDS PAGE (Fig. 10). These experiments showed that most HNK-1<sup>+</sup>/L2<sup>+</sup> molecules were located in a broad band or bands between 160 and 180 kD, although weaker labeling was seen at higher molecular weights and in faint bands at 140 and 100 kD (which are difficult to see in Fig. 10). Anti-J1 serum also mainly labeled a broad band between 160 and 180 kD, with weaker labeling of a number of bands at and above 200 kD, and in the region



**Figure 8.** Immunogold labeling of a transverse ultrathin frozen section of optic nerve with HNK-1 antibody. After labeling, the sections were stained with aqueous uranyl acetate and then embedded in methylcellulose. A node of Ranvier (AX, nodal axon; M, paranodal oligodendrocyte loop) can be seen adjacent to a large astrocyte process (TA). Note the heavy labeling in the paranodal region (open arrows) where perinodal astrocyte processes would be expected to be, and the lack of labeling of the transverse astrocyte process (TA). The former processes are poorly preserved with this embedding technique. Bar, 0.5  $\mu\text{m}$ .



**Figure 9.** Immunogold labeling of two different transverse ultrathin frozen sections of optic nerve with L2 (a) and RT97 (b) antibodies. After labeling, the sections were osmicated and embedded in Epon. Both sections show nodes of Ranvier (PA, perinodal astrocyte process; Ax, nodal axon; M, paranodal oligodendrocyte loop). Note that whereas the L2 antibody labeling is mainly on the surface of the perinodal astrocyte processes (PA in a), the RT97 antibody labeling is largely confined to the interior of the large axon (A in b). Bar, 0.25  $\mu\text{m}$ .



**Figure 10.** Autoradiography of adult optic nerve proteins after SDS PAGE, transfer to nitrocellulose, and immunolabeling as described in Materials and Methods. Molecular mass markers are shown on the left (in kilodaltons). The lanes were labeled with (a) anti-N-CAM serum, (b) anti-L1 serum, (c) L2 antibody and (d) anti-J1 serum. Note that both L2 and J1 antibodies label mainly a broad band(s) between 160 and 180 kD, whereas N-CAM and L1 antibodies label bands at about 180, 140, and 120 kD, and 200 and 140 kD, respectively.

of 60–65 kD; the latter bands are probably degradation products whereas the weak staining at 200 kD may represent persistence of the higher molecular weight form of J1 described in younger brains (16). N-CAM and L1 antisera only labeled bands at 180, 140, and 120, and at 200 and 140 kD, respectively, as found in other studies (26, 30).

## Discussion

In immunofluorescence studies of semithin frozen sections of adult rat optic nerve we have found that the HNK-1 and L2 monoclonal antibodies label most intensely around the nodes of Ranvier. In double-labeling experiments, these HNK-1<sup>+</sup>/L2<sup>+</sup> structures were shown to be stained by anti-GFAP but not by anti-NF antibodies, suggesting that the staining was associated with perinodal astrocyte processes. This was confirmed in immunogold electron microscopic studies of ultrathin frozen sections, which showed that these antibodies bound mainly to the perinodal astrocyte processes. Since astrocyte cell bodies and other processes were not labeled by either monoclonal antibody in these ultrastructural immunogold studies, we conclude that HNK-1<sup>+</sup>/L2<sup>+</sup> molecules are highly concentrated on perinodal astrocyte processes.

In contrast to the immunogold studies in which labeling was confined to the perinodal area, immunofluorescence studies also revealed weak staining of fine, longitudinally orientated processes between axons. This additional labeling may reflect increased sensitivity of the immunofluorescence technique. Some, but not all, of these longitudinally orientated processes were GFAP<sup>+</sup> and could occasionally be seen to extend into nearby nodes; in these cases, staining with HNK-1 and L2 antibodies became much more intense in the region of the node, confirming that the HNK-1/L2 antigen was highly concentrated around perinodal astrocyte processes. Even by immunofluorescence, the antigen was not detected over astrocyte cell bodies or large astrocyte processes, suggesting that the weak labeling of the fine longitudinal GFAP<sup>+</sup> processes was due to relatively low concentration of the HNK-

1/L2 antigen in or on longitudinal astrocyte processes that are continuous with the perinodal processes. The labeled GFAP<sup>+</sup> processes might have been astrocyte processes containing insufficient GFAP to be detectable by immunofluorescence, or longitudinal processes from other glial cells, such as oligodendrocytes. The weak labeling of oligodendrocyte paranodal loops in the immunoelectron microscope studies suggests that oligodendrocytes express the HNK-1/L2 antigen *in vivo*; these cells have been shown to express the antigen *in vitro* (23, 42).

What is the nature of the HNK-1<sup>+</sup>/L2<sup>+</sup> molecules associated with perinodal astrocyte processes? Previous studies have shown that the HNK-1 and L2 antibodies recognize a carbohydrate determinant on various glycoproteins, including MAG, N-CAM, and the L1 and J1 glycoproteins, although apparently only a subset of these glycoproteins expresses the epitope (17). The sensitivity of the labeling to periodate in our experiments is consistent with the antigenic determinant on the nodal astrocyte processes being a carbohydrate. Two lines of evidence suggest that one of the HNK-1<sup>+</sup>/L2<sup>+</sup> molecules associated with perinodal astrocyte processes is the J1 glycoprotein, a recently described 160-kD molecule, found on the surface of oligodendrocytes and astrocytes in culture, which expresses the HNK-1/L2 determinant and appears to be a novel cell adhesion molecule (16). First, by immunofluorescence the patterns of binding of anti-J1 serum and the HNK-1 and L2 monoclonal antibodies were the same. Second, in immunoblotting experiments on proteins extracted from adult optic nerve, all three antibodies mainly labeled a broad band at 160–180 kD. Although we cannot exclude the possibility that there are other HNK-1<sup>+</sup>/L2<sup>+</sup> molecules associated with astrocyte processes at the node of Ranvier, it seems unlikely that MAG or the L1 glycoprotein is concentrated there since they are thought to be confined in the central nervous system to oligodendrocytes and/or myelin (36) and to neurons (30), respectively. Moreover, although N-CAM antiserum brightly labeled large astrocyte processes throughout the nerve it did not label perinodal astrocyte processes, suggesting that N-CAM is not concentrated on these processes. The presence of N-CAM on astrocytes has been previously demonstrated both *in vivo* (19) and *in vitro* (14, 26).

If the HNK-1 and L2 antibodies recognize MAG, N-CAM, and L1, why did they not stain other structures in the optic nerve, such as axons and myelin? Since it has been shown that only a minority of N-CAM and L1 molecules are recognized by the HNK-1 and L2 antibodies (17), it is possible that most of the N-CAM and L1 molecules present in the adult optic nerve are HNK-1<sup>-</sup>/L2<sup>-</sup>. Our immunoblotting experiments support this possibility since the bands labeled by either anti-N-CAM or anti-L1 sera were labeled only weakly, if at all, by the HNK-1 and L2 monoclonal antibodies. This was particularly striking in the case of the 120 kD N-CAM band, which appeared to be the major form of N-CAM in the adult optic nerve. Since HNK-1 and L2 antibodies only weakly labeled a band at 100 kD where MAG would be expected (28), it is possible that only a minority of MAG molecules in the optic nerve are HNK-1<sup>+</sup>/L2<sup>+</sup>, which would explain why these antibodies did not stain myelin. The weak staining of paranodal oligodendrocyte loops by HNK-1 and L2 antibodies in ultrathin sections could have been due to binding to either MAG or J1, since anti-J1 serum has been shown to

label oligodendrocytes in vitro (16).

The presence of perinodal astrocyte processes encircling the exposed axon has been well described in nodes of Ranvier in spinal cord (9, 29) and optic nerve (11, 40). To our knowledge, this is the first demonstration that the plasma membrane of these processes is biochemically specialized. Given the specialized structure and function of nodes, this finding is perhaps not surprising, especially since it is not the only example of regional specialization of the astrocyte cell surface. Characteristic orthogonal arrays of intramembranous particles seen in freeze-fracture electron microscopy are highly concentrated in astrocyte plasma membranes where they contact blood vessels or the pial surface (18); although these arrays are also seen in perinodal astrocyte membrane, they appear not to be concentrated there (40). Biochemical specialization of astrocyte processes associated with blood vessels has also been observed, with these processes having a high concentration of Na<sup>+</sup>-K<sup>+</sup>-ATPase (44), and high alkaline phosphatase activity (5).

Our finding that the J1 glycoprotein is concentrated on perinodal astrocyte processes has potentially important functional implications in view of the previous finding that anti-J1 antibodies inhibit neuron to astrocyte (but not neuron to neuron) adhesion in vitro (16). Taken together, these in vivo and in vitro observations raise the strong possibility that this molecule plays an important part in the neuron-astrocyte interactions that presumably are involved in the assembly and/or maintenance of the node of Ranvier.

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