Basal Lamina Formation at the Site of Spinal Cord Transection

Earl R. Feringa, MD, Timothy F. Kowalski, BS, and H. Lee Vahlsing, MS

The pia-glial basal lamina (BL) at the site of spinal cord injury could be an important physical impediment to central nervous system regeneration. We used an epithelial BL-specific immunohistochemical stain to determine the location of the pia-glial BL after spinal cord transection.

Small segments of BL were found at the margin of the lesion 5 days after transection. After 10 days, longer and more numerous segments were seen. At 20 days, the entire transected end of the spinal cord was capped by a layer of BL.

Feringa ER, Kowalski TF, Vahlsing HL: Basal lamina formation at the site of spinal cord transection. Ann Neurol 8:148–154, 1980

The commonly held conception that axons of the mammalian central nervous system (CNS) cannot regenerate because of an intrinsic inability to grow has been refuted by experiments demonstrating that CNS neurites can change connections, sprout collaterals, and grow into areas outside the usual field of their processes [7]. Recent experiments in rats have even shown regeneration of long ascending or descending spinal cord axons over distances of at least 2 to 3 cm [3–6]. In each case, however, the outgrowth of new axons or collateral sprouts in mammals has been meager. Also, those axons which did regrow have failed to reestablish connections that would restore useful function to the organism.

The identity of the factor or factors that prevent or limit regrowth of long spinal cord tracts and reestablishment of connections useful to the mammalian organism remain obscure. Older research focused on the glial scar as a mechanical block to regenerating axons. In the injured newt and tadpole optic nerve, however, a dense glial scar appears to provide no detectable impediment to regrowth of retinal axons to the tectum [10, 12]. Electron micrographs of the scarred tissue demonstrate that regrowing axons have no difficulty penetrating the glial scar as they push aside glial processes and reestablish their central connections.

In an effort to identify in greater detail anatomical factors that might play a role in guiding or blocking regenerating axons, several investigators have studied spinal cord injury sites under the electron microscope. Some of these studies [2, 8] have suggested that the basal lamina (an ultrastructural component of the more familiar light microscopic basement membrane) may cap the cut end of a transected mammalian spinal cord and be an important physical barrier to CNS axonal regeneration.

Basal lamina (BL) is composed of type IV collagen embedded in an amorphous matrix consisting of an acid mucopolysaccharide and various glycans, all associated with some noncollagen glycoproteins. Integrity of the BL appears to be necessary for maintenance of orderly tissue structure as well as for healing of lesions in many organs and tissues [14]. In CNS, the BL is located at the pia-glial interface and at the vascular-CNS interface.

Electron microscopic studies of this structure are limited by the small field that can be viewed effectively. A time study of the extent of capping by BL after spinal cord injury would be very time consuming by electron microscopy and might not yield reliable estimates for the total area of injury.

This report describes the use of a highly specific immunohistochemical stain to demonstrate development of the pia-glial BL in scar formed at the site of transected rat spinal cord at various intervals after injury.

Materials and Methods

The animals used in this experiment were adult albino isogeneic female rats which were originally derived from Wistar stock. Groups of 3 rats each were perfused at 0, 3, 5, 7, 10, 15, and 20 days after spinal cord transection. The spinal cord transection technique was identical to that used

From the Departments of Neurology and Pathology, Ann Arbor Veterans Administration and University of Michigan Medical Centers, Ann Arbor, MI.

Received July 23, 1979, and in revised form Dec 7. Accepted for publication Dec 13, 1979.

Address reprint requests to Earl R. Feringa, MD, Chief, Neurology Service (127), Veterans Administration Medical Center, 3350 La Jolla Village Dr, San Diego, CA 92161.

 0364-5134/80/080148-07$01.25

in previous experiments [4]. Postoperatively, the animals' bladders were emptied every eight hours by the method of Credé until reflex control returned.

The complete brain and spinal cord of rats from our inbred colony were cleared of blood and the dura coverings were removed. The pial surface was carefully preserved and included with the CNS tissue. This tissue was homogenized and treated with various enzymes, detergents, and physical disruptions. A final centrifuged pellet was observed with the electron microscope. Approximately 30% of the pellet was identifiable as fragments of BL; the rest consisted of collagen and amorphous material.

This pellet, suspended in Freund's complete adjuvant, was injected subcutaneously into a New Zealand white rabbit. Antigenic challenges were repeated at two and four weeks after the initial injection. Rabbit hyperimmune serum was then purified by adsorption on splenic pulp to yield an epithelial BL-specific serum. Since the spleen contains endothelial BL and collagen, the antibodies specific for these antigens were eliminated by this exhaustive adsorption step. The specific (adsorbed) serum was used in a modification of the peroxidase-antiperoxidase (PAP) indirect antibody technique [13] to label pia-glial BL. Complete details of this staining technique are published elsewhere [9].

For each animal the transection site was sampled at 100 μ intervals. Stained longitudinal sections were examined by both bright field (1,000× magnification) and dark field (400× magnification) microscopy. The evaluator did not know from which experimental group the tissue came. Linear deposition of granular material was the criterion for positive staining.

Results

On day 0, sections showed an accumulation of red blood cells (RBCs) in the lesion site. The cranial and caudal spinal cord stumps were completely detached from each other. No connective tissue components were seen. Staining in these sections was limited to the normal BL at the pia-glial interface adjacent to intact CNS tissue.

The 3-day sections showed few RBCs in the transection site. The cranial and caudal CNS stumps remained apart, but some connective tissue components were present in the gap. Again, staining was confined to the RBCs and the normal BL.

At 5 days, sections showed only occasional RBCs in the transection site. Occasional bridging of the transection gap by connective tissue elements was seen. Short linear segments of a granular, staining substance were present at the CNS–connective tissue margins within the lesion site. This stained layer was the transection site BL. Staining of RBCs and normal BL was also seen (Fig 1A).

RBCs were not found in the 7-day sections. Many of these sections showed the transection site bridged by connective tissue elements. These sections showed more of the short stained segments of transection site BL than the 5-day sections. The normal BL stained as well.

The transection sites of almost all the 10-day sections were bridged by connective tissue elements. The segments of transection site BL were as numerous as in the 7-day group; however, each segment was longer. The normal BL continued to stain (Fig 1B).

The transection gaps of the 15-day sections were filled by connective tissue bands which were parallel to the ends of the stumps. The normal BL also stained. The transection site BL was almost a continuous staining band along the CNS connective tissue margins.

The 20-day sections were similar to the 15-day sections except that the transection site BL staining band may have been wider (Fig 1C).

The amount of transection site BL varied somewhat among animals within a particular time group; however, each group as a whole was distinct from the others.

Discussion

Staining Specificity

The stain used in this experiment was previously tested on BL of different embryological and functional derivations [9]. The specificity of the stain was confined to epithelial BL regardless of germ layer origin. Electron microscopy showed that the stain's complexes localized on the pia-glial BL only and not on adjacent connective tissue components. Also, antigenic and methodological negative controls showed no staining of the pia-glial BL of the spinal cord (Figs 2, 3).

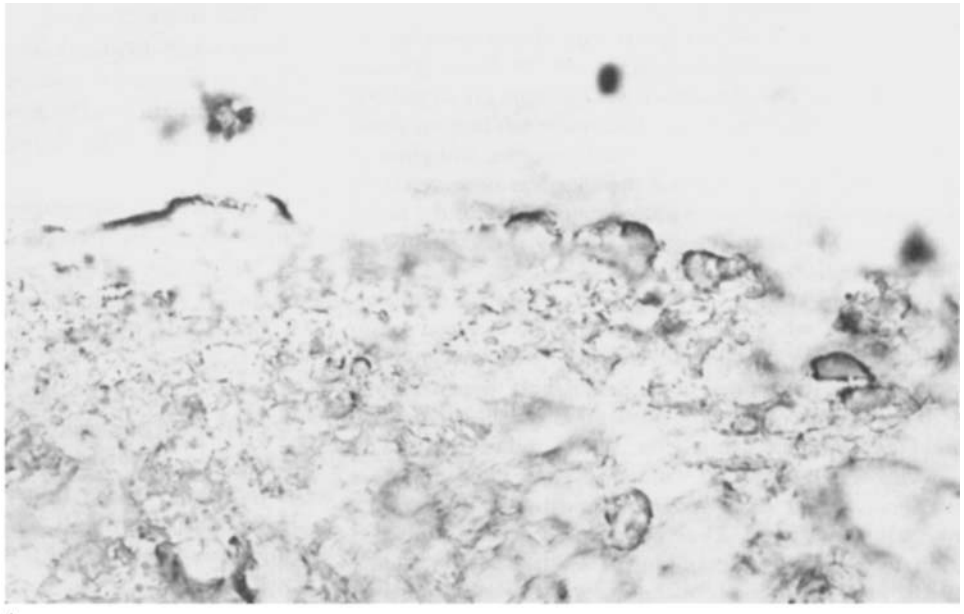
Some background staining did occur in this experiment. RBCs, when present, and endogenous tissue peroxidase activity were the contributing factors. However, this background staining was easily distinguished from the linear deposits which constitute the BL stain.

In this experiment, the areas of normal CNS tissue on each section acted as a positive control for the BL stain. One could determine the intensity of background staining along with the degree of specific staining by viewing the normal BL areas on each slide.

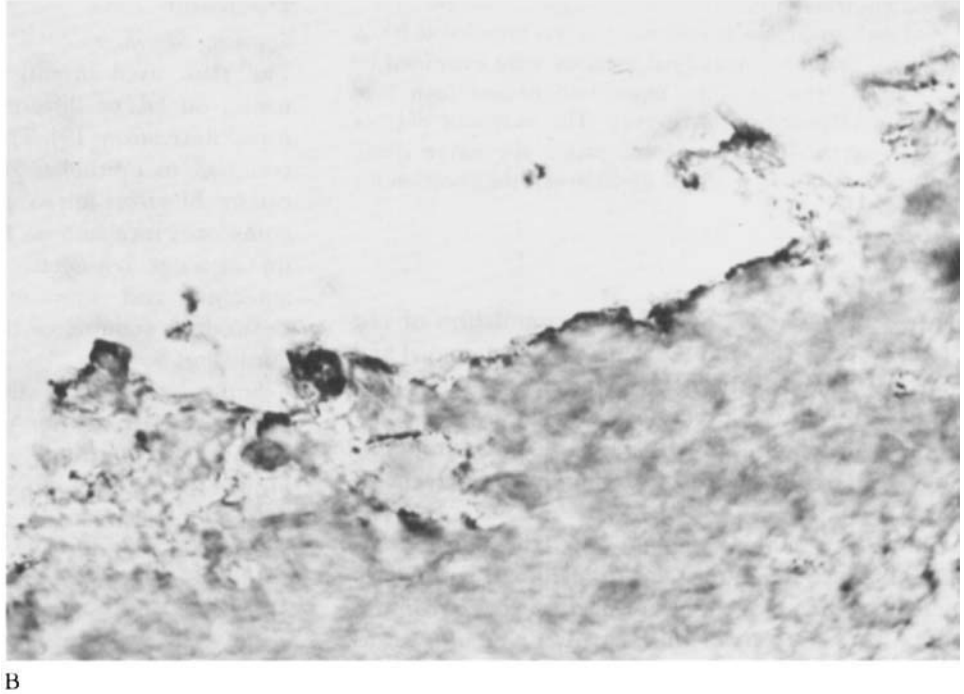
BL Margination Phenomenon

The results of this study indicate that BL* quickly forms a boundary at the CNS tissue margins after injury. At 5 days, long before light microscopic evidence of reactive gliosis is present, the boundary

*In this experiment, the presence of substances containing BL antigens, as detected by the immunohistochemical stain, are taken as evidence for the presence of BL.



A

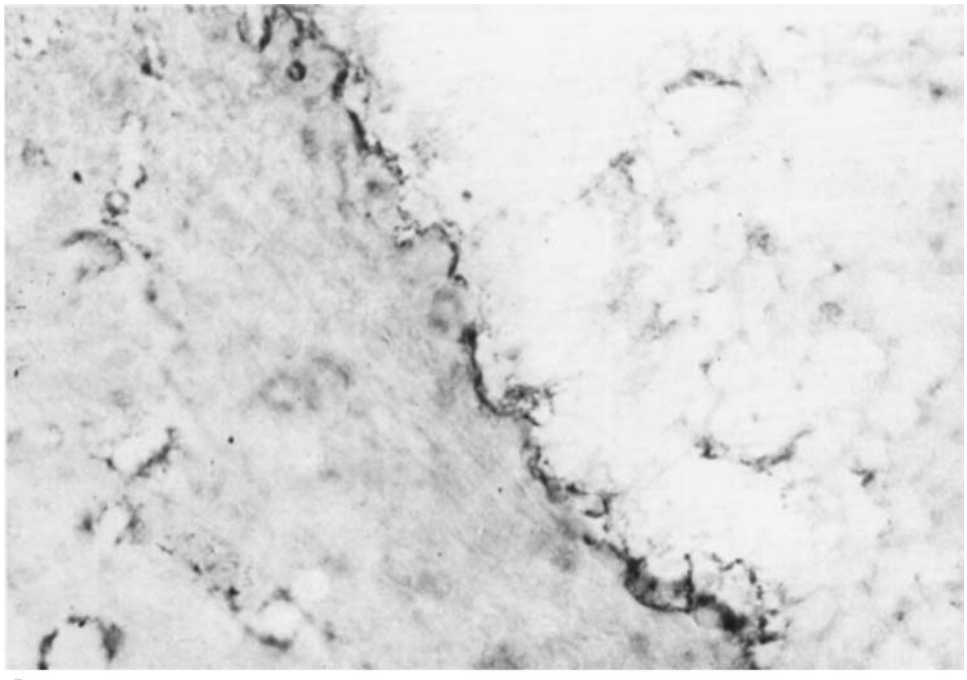


B

Fig 1. Bright-field microscopy at different intervals after spinal cord transection. (A) At 5 days short linear segments of basal lamina are found on the CNS tissue margins in the lesion site. (B) Ten days after transection, longer linear segments of basal lamina are seen on the CNS tissue margins in the lesion site. (C) By 20 days the entire tissue margin at the lesion site is capped by a basal lamina layer. (Epithelial basal lamina PAP stain; ×800 before 25% reduction.)

between viable CNS and early collagenous scar shows segments of BL. By 10 days this is much more continuous. By 20 days the entire transected end of the spinal cord is capped by a layer of BL.

What is the significance of this capping phenomenon? Stensaas and Feringa [12], using a freeze lesion of newt optic nerve, observed that a preserved tube of BL appeared to provide a guide for regenerating optic nerve axons. Freezing the optic nerve destroyed all cellular profiles in cross sections of the



C

injury studied with the electron microscope at 7 days. Only an occasional macrophage was seen in the mass of necrotic tissue contained in the optic sheath. In spite of such massive destruction, the tube of BL remained uninterrupted at this stage and also at 10, 14, 21, and 31 days following injury. As retinal axons grew out toward the tectum, all were contained within (and possibly guided by) this tube of BL. None penetrated it; none got lost along other tissue planes as occurs after optic nerve surgical transection in tadpoles, which disrupts the tube of BL [11].

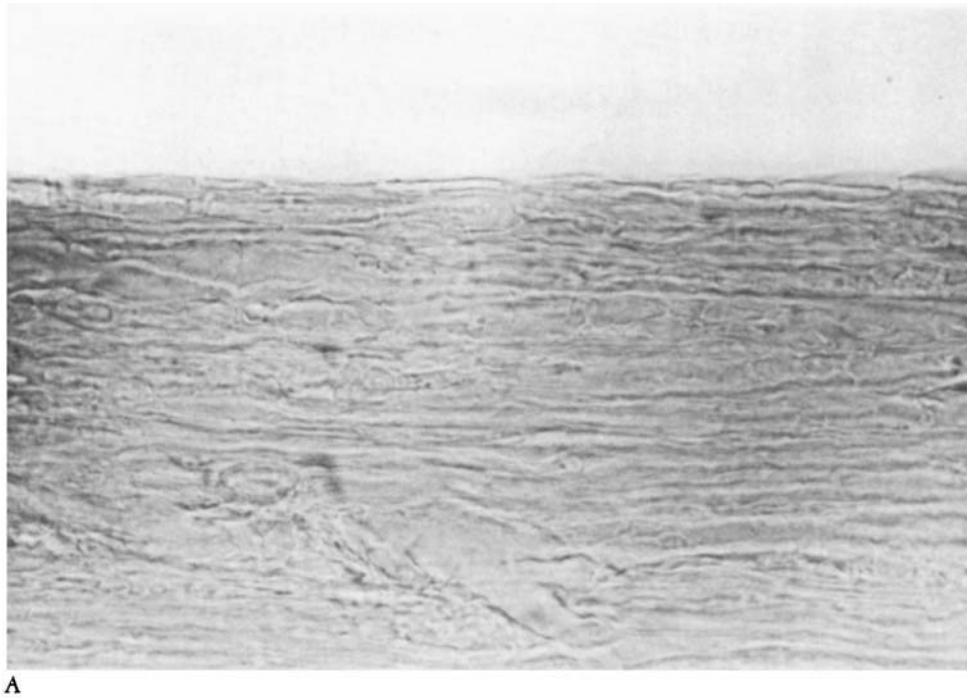
Kao et al [8] have studied the spinal cord injury site after transection in dogs. While their focus was on the surgical technique they use to assist regrowth of axons, their electron micrographs showed that some regenerating axons appeared to be turned back by a sheet of BL. Some such axons made a 180-degree turn after contacting this ultramicroscopic structure. They suggested that the BL may cap the end of the cord within two weeks to one month after transection.

Bernstein et al [2] studied the hemisected rat spinal cord and noted that at 7 days a BL was "reestablished and followed the topography of the finger-like projections of the pathological spinal cord." All nerve fibers were central to the BL. At 30 days postoperatively conglomerates of small, bouton-like terminal portions of axons containing many mitochondria and flattened, spherical or granular vesicles were located immediately rostral to the BL in the spinal cord. These profiles had some of the morphological characteristics which they had found in goldfish when spinal cord regeneration was thwarted by an implanted Teflon block [1].

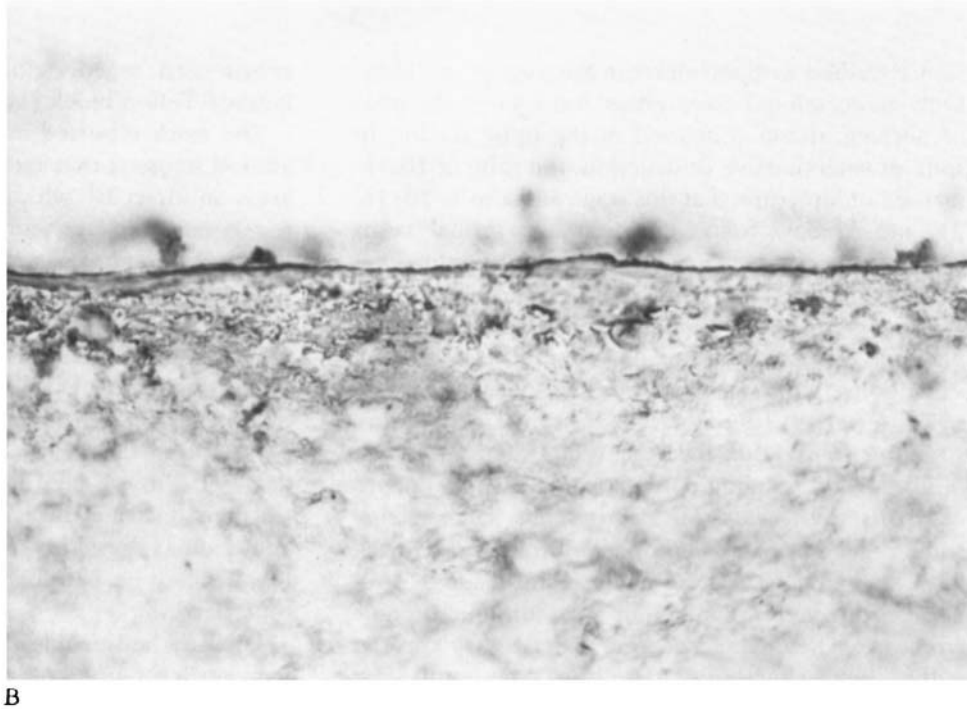
The work reported in this paper and that just reviewed suggests that axons cannot, or at least *do* not, cross an intact BL with ease. The time delay before regeneration begins and the rate of regeneration in mammals are not known, inasmuch as such regeneration cannot be reliably reproduced in long tracts. In newt optic nerve kept at 20°C, regeneration begins at 10 to 14 days and is substantial by 30 days [12]. It is not possible to estimate reliably the delay in start of regeneration in mammals by extrapolating from poikilotherms, but available evidence suggests a delay of 7 to 14 days before regeneration begins. If the path of such axons is already blocked by a new BL and if axons cross this structure with great difficulty, the BL may be partly responsible for the failure of regeneration.

It seems reasonable that axons would take a few days to "gear up" before they try to grow across a transection site. The second operation that Kao et al [8] performed one week after the injury carefully removed scar and necrotic debris at the site of transection. Perhaps in the process they also removed parts of the BL which by one week had begun to cap the cord. Under these circumstances a few regenerating axons, having already geared up to grow, could cross the gap before a new BL develops, accounting for the successful axonal regeneration they reported.

Our success with cyclophosphamide treatment [3, 6] might be due to a nonspecific effect of that agent on the production of BL. Even a minor delay in



A

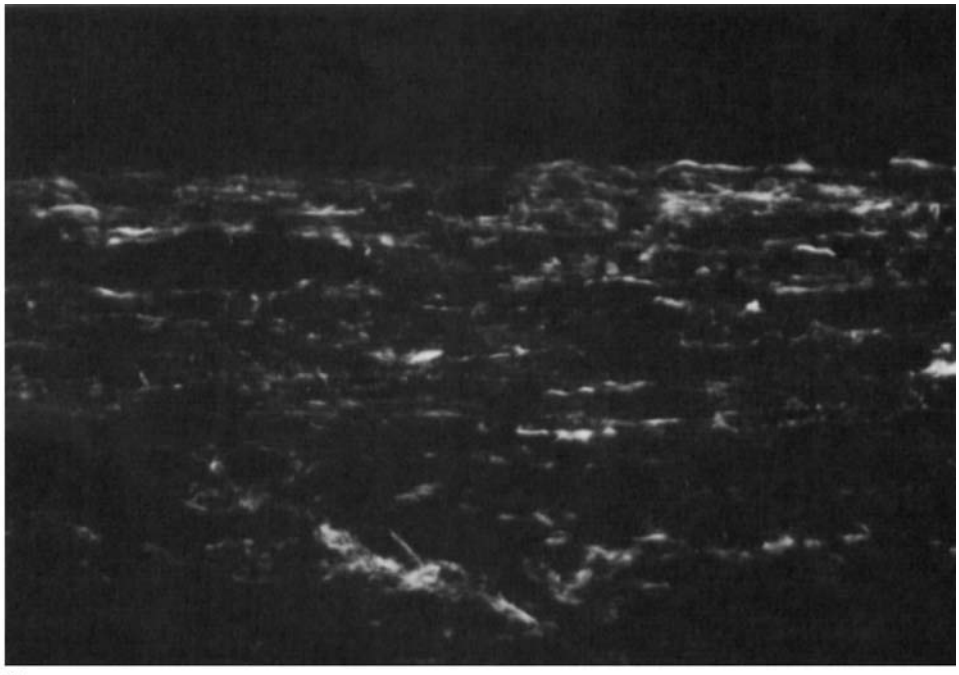


B

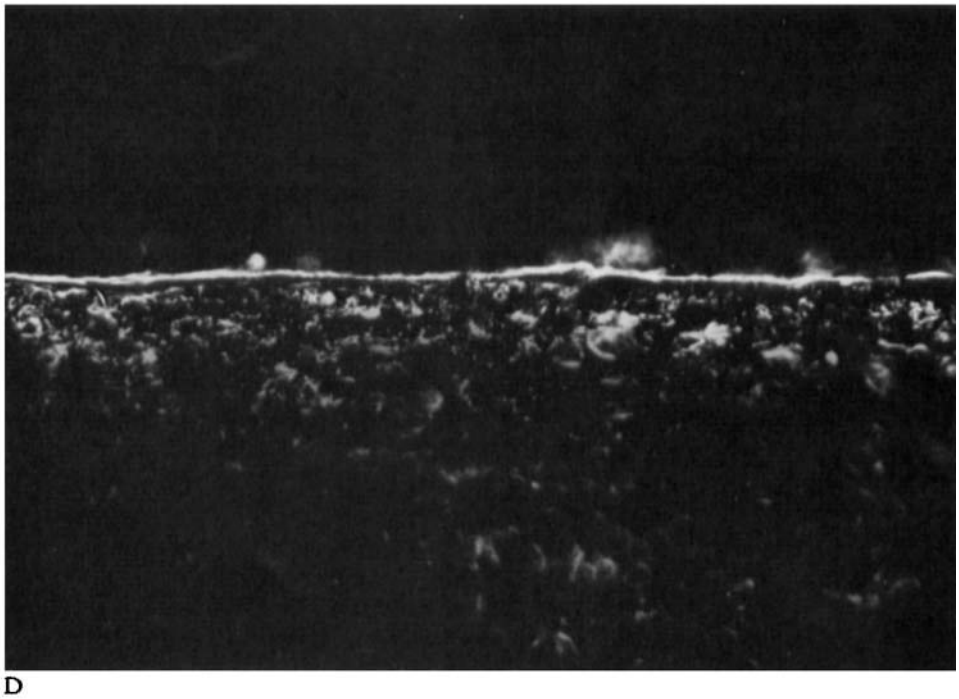
Fig 2. Bright-field and dark-field micrographs. (A,C) Negative control slides. The pia-glial basal lamina is unstained. These slides were prepared in a manner identical to that used for B and D except that the rabbit antirat BL hyperimmune serum was first adsorbed on the BL fraction of CNS tissue originally used to stimulate production of antibodies in the rabbit. (B,D) Normal spinal cord showing pia-glial BL-specific stain. (Epithelial basal lamina PAP stain; ×800 before 25% reduction.)

production of BL, if accompanied by no additional delay in commencement of axonal sprouting, might provide an opportunity for a few axons to grow across the site of spinal cord transection, giving evidence of long tract regeneration in cyclophosphamide-treated animals.

The success we have reported in rats made immunologically unresponsive to CNS antigens [5] would, however, remain unexplained. Only if pretreatment



C



D

by neonatal exposure to CNS antigens altered the production of BL at the site of cord injury would we be able to attribute the failure of regenerating axons to cross an injury site to the same anatomical mechanism. This seems unlikely. Even though the BL lies at the anatomical interface between the immunologically distinct CNS and the rest of the organism, we know of no evidence to suggest that it is produced by an immunological mechanism or is changed in states of altered immunological responsiveness. Nonetheless, we intend to study what effect our treatments have on the temporal development of the BL cap after spinal cord transection in rats.

The fact that BL caps the cut end of the spinal cord does not prove that this cap blocks regenerating axons. Further experiments are necessary to determine if the cap is itself an impediment to CNS axonal regeneration, or simply an epiphenomenon.

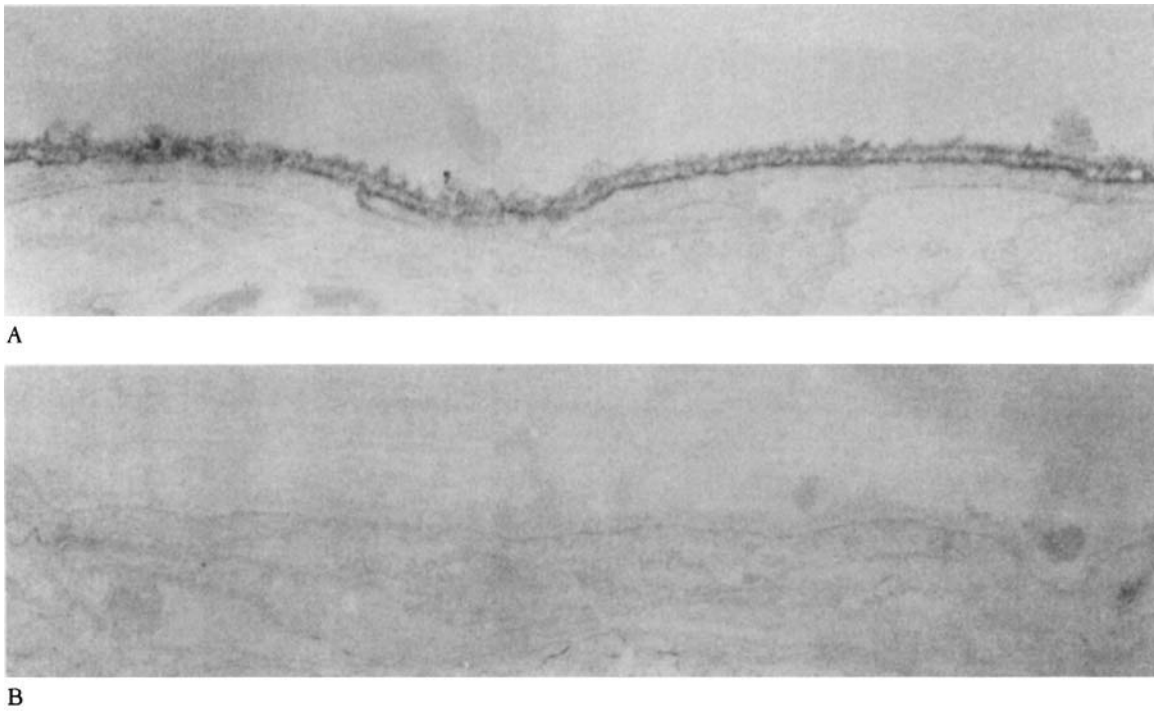


Fig 3. Electron micrographs of immunohistochemical stain. (A) Localization of PAP complexes on the rat pia-glial BL using a splenic adsorbed specific serum. (B) PAP complexes are absent on rat pia-glial BL when the goat antirabbit IgG linking step of the PAP staining technique was omitted. (×30,000.)

Supported by institutional research support to the Veterans Administration Medical Centers, Ann Arbor, MI, and San Diego, CA, and by development funds of the Department of Pathology, University of Michigan Medical Center.

The authors acknowledge the services provided by Mr Robert McKnight and Ms Linda Lee Austin for photography, histology, and technical assistance, and the help of Mrs Diane Trakas and Barbara Reader in manuscript preparation.

References

1. Bernstein JJ, Bernstein ME: Ultrastructure of normal regeneration and loss of regenerative capacity following Teflon blockage in goldfish spinal cord. Exp Neurol 24:538–557, 1969
2. Bernstein JJ, Wells MR, Bernstein ME: Effect of puromycin treatment on the regeneration of hemisected and transected rat spinal cord. J Neurocytol 7:215–228, 1978
3. Feringa ER, Davis SW, Vahlsing HL, et al: Fink-Heimer/Nauta demonstration of regenerating axons in the rat spinal cord. Arch Neurol 35:522–526, 1978
4. Feringa ER, Gurden GS, Strodel W, et al: Descending spinal motor tract regeneration after spinal cord transection. Neurology (Minneap) 23:599–608, 1973
5. Feringa ER, Nelson KR, Vahlsing HL, et al: Spinal cord regeneration in rats made immunologically unresponsive to CNS antigens. J Neurol Neurosurg Psychiatry 42:642–648, 1979
6. Feringa ER, Shuer LM, Vahlsing HL, et al: Regeneration of corticospinal axons in the rat. Ann Neurol 2:315–321, 1977
7. Guth L: History of central nervous system regeneration research. Exp Neurol 48:no 3, part 2:3–15, 1975
8. Kao CC, Chang LW, Bloodworth JMB: Axonal regeneration across transected mammalian spinal cords: an electron microscopic study of delayed microsurgical nerve grafting. Exp Neurol 54:591–615, 1977
9. Kowalski TF, Vahlsing HL, Feringa ER: Light microscopic, immunohistochemical localization of the pia-glial basal lamina. J Histochem Cytochem 8:347–353, 1980
10. Reier PJ: Penetration of grafted astrocytic scars by regenerating optic nerve axons in *Xenopus* tadpoles. Brain Res 164:61–68, 1979
11. Reier PJ, Webster HdeF: Regeneration and remyelination of *Xenopus* tadpole optic nerve fibers following transection or crush. J Neurocytol 3:591–618, 1974
12. Stensaas LJ, Feringa ER: Axon regeneration across the site of injury in the optic nerve of the newt *Triturus pyrrhogaster*. Cell Tissue Res 179:501–516, 1977
13. Sternberger LA, Hardy PH, Cuculis JJ, et al: The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase–anti-horseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 18:315–333, 1970
14. Vracko R: Basal lamina scaffold-anatomy and significance for maintenance of orderly tissue structure. Am J Pathol 77:314–346, 1974