

Expression of polysialylated neural cell adhesion molecule (PSA-N-CAM) in developing, adult and regenerating caudal spinal cord of the urodele amphibians

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ABSTRACT The patterns of expression of polysialylated («embryonic») form of Neural Cell Adhesion Molecule (PSA/E-N-CAM) and of all N-CAM isoforms were investigated by indirect immunofluorescence and immunoblotting during the development of the Central Nervous System (CNS) and during the regeneration of the caudal Spinal Cord (SC) of the amphibian urodeles *Pleurodeles waltl* (Pw) and *Notophthalmus viridescens* (Nv). In this study, a monoclonal antibody to group B *Meningococcus* (anti-Men-B) which recognizes alpha-2,8-linked sialic units of PSA-N-CAM, and polyclonal anti-total N-CAM antibodies were used. Total-N-CAM immunoreactivities were consistently detected throughout the CNS of developing and adult newts. PSA-N-CAM expression predominated in «embryonic» developing CNS and was reduced to certain CNS areas in the adult urodeles. In the case of SC, the expression level of this isoform of N-CAM dramatically decreased to become low and nearly restricted to some ependymoglia cell surfaces. Interestingly, during newt tail regeneration, PSA-N-CAM was intensely reexpressed in regenerating SC, at the surface of ependymoglia cell processes and in axonal compartments. Expression was maximal at 4 to 6 weeks following amputation, and then gradually returned to a normal adult low level in well differentiated SC. These findings strongly supported the view that the expression of PSA-N-CAM was associated with the properties of plasticity shown by the SC ependymoglia tissue in newts, during tail regeneration. On the other hand, the high level of PSA-N-CAM expression in axonal compartments of regenerating as well as developing SC suggested that these isoforms of N-CAM could be implicated in axonal outgrowth within the «tunnels» defined by the radial ependymoglia processes. This transient PSA-N-CAM expression could therefore be considered both as a negative modulator of cell-cell and cell-substrate interactions and as a permissive factor for neuron differentiation.

KEY WORDS: *polysialylated N-CAM, regeneration, spinal cord, urodele amphibians*

Introduction

In some lower vertebrates (i.e. certain fish and amphibian urodeles), adult animals have extensive regeneration abilities of the CNS, in contrast with reactive gliosis and aborted neuritic outgrowth observed in mammals (Reier and Roule, 1987). In particular, urodeles are characterized by epimorphic regeneration of their missing or amputated appendages (limbs and tail). During regeneration following newt tail amputation, tissues are reconstructed by direct budding from tissues of the stump (Holtzer, 1956). The most striking aspect of this system lies in the plasticity or remodelling properties of the SC, which is able to differentiate a new structure within the regenerate.

Histological observations have shown that glioependymal and astroglial cells, which emerge from the amputated spinal cord, form an ependymal tube which guides axon growth in a rostro-caudal

direction (Egar and Singer, 1972; Nordlander and Singer, 1978). Within the wall of this tube, ependymal cells are able to divide (Singer *et al.*, 1979; Arsanto *et al.*, 1992) and to differentiate in supportive cells and in neuronal cells. The regenerated ependymal tube has thus the capacity to re-build a cytoarchitecture which provides a guiding substrate for axon outgrowth and a stereotyped motoneuron positioning sketch. A segmented organization pattern with ventral root exits and spinal ganglia formation takes place

Abbreviations used in this paper: Nv, *Notophthalmus viridescens*; Pw, *Pleurodeles waltl*; SC, spinal cord; CNS, central nervous system; PNS, peripheral nervous system; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; PSA, polysialic acid; PSA-N-CAM, polysialylated neural cell adhesion molecule; Anti-Men-B, monoclonal antibody to capsule PSA units of *Meningococcus B*; anti-total N-CAM, polyclonal antibodies against all N-CAM isoforms; endo-N, endoneuraminidase.

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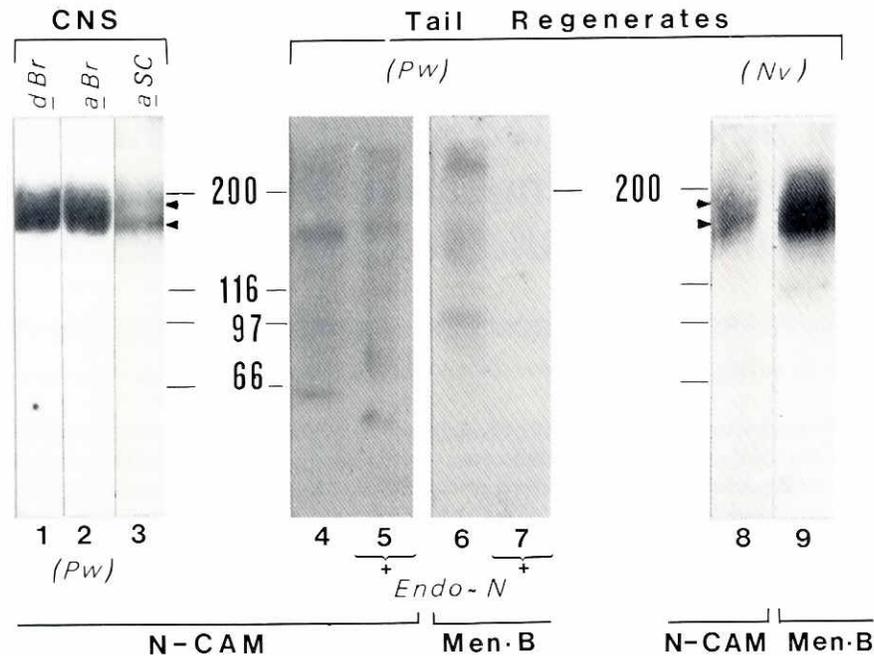


Fig. 1. Western blot analysis of total N-CAM and PSA/E-N-CAM in samples of CNS and tail regenerates from adult Pw (lanes 1-7) and Nv (lanes 8-9). The blots were probed with anti-total N-CAM (lanes 1-5 and 8) or anti-Men-B antibodies (lanes 6-7 and 9). A diffuse material with a range of 140-220 kDa is recognized by the anti-chicken total N-CAM in extracts from developing/larval (lane 1: dBr) and adult (lane 2: aBr) Pw brain. In adult SC extract from Pw (lane 3: aSC), two faint bands at 140 and 180 kDa (arrowheads) are visible in diffuse component. In extract from 3-week-old regenerate of Pw (lane 4), a diffuse staining extending from a large band at 140 kDa (arrowhead) to 250 kDa is observed. This reactivity is slightly decreased but not eliminated by endo-N treatment (lane 5). (Lanes 6-7) Immunodetection of PSA-N-CAM in 3 week-old tail regenerates of Pw. Anti-Men-B MAAb recognizes a diffuse material, especially with a range of 200-250 kDa (lane 6), which is removed by endo-N (lane 7). (Lanes 8-9) Immunodetection of total N-CAM (lane 8) and PSA-N-CAM (lane 9) in 3 week-old tail regenerates of Nv. In these extracts, total N-CAM reactivity appears as faint bands at 140-180 kDa (arrowheads) and a diffuse staining extending from these bands and beyond 200 kDa (lane 8) and PSA-N-CAM reactivity as a diffuse material with a range of 140-250 kDa (lane 9). Molecule mass standards: Myosin (200 kDa), galactosidase (116 kDa), phosphorylase b (97 kDa) and bovine serum albumin (66 kDa).

according to a rostro-caudal gradient (Géraudie et al., 1988; Arsanto et al., 1992).

During vertebrate development and some processes of regeneration, it was demonstrated that the Neural Cell Adhesion Molecule (N-CAM) was involved in many histogenetic events (Rutishauser et al., 1978; Thiéry et al., 1982; Grumet et al., 1984; Edelman, 1985). Its conversion from a polysialylated or «embryonic» (PSA-N-CAM or E-N-CAM) to an «adult» form with a lower number of sialic acid residues, plays a major role in development of the nervous system (Rutishauser, 1989). The extent of N-CAM glycosylation has been suggested to regulate functions of this molecule (Edelman, 1986). The polysialylated («embryonic») form has been shown to be less adhesive than the «adult» form (Hoffman and Edelman, 1983; Sadoul et al., 1983; Chuong and Edelman, 1984; Crossin et al., 1984). Moreover, the presence of PSA could provide broad steric effects (Yang et al., 1992), modulating both cell-cell and cell-substrate interactions *in vitro* (Acheson et al., 1991) as well as *in vivo* (Landmesser et al., 1990). It is thus likely that the conversion from PSA-N-CAM isoform to N-CAM corresponds to a loss of plasticity and to a stabilization of adhesion. In agreement with this view, it had been shown that in the brain of adult mammals, PSA-N-CAM remains expressed in well-defined areas where it may be involved in tissue plasticity and cell reshaping under physiological conditions (Miragall et al., 1988; Aaron and Chesselet, 1989; Bartsh et al.,

1990; Goldowitz et al., 1990; Theodosis et al., 1991; Bonfanti et al., 1992).

The main question we addressed here to the urodele amphibian system deals with the tissue plasticity of the SC consistent with the recovery of histogenetic properties during regeneration. According to the important morphoregulator role attributed to the PSA-N-CAM in mechanisms of neuroplasticity mentioned above, we postulated that modulations of the expression of this N-CAM isoform could also participate in controlling cell adhesion during the CS regeneration in newts. Characterization of newt N-CAM was previously provided by Maier et al. (1986) in *Notophthalmus viridescens* (Nv) and by Saint-Jeannet et al. (1989) in *Pleurodeles waltli* (Pw). In adult brain of Nv, the presence of alpha-2,8 linked polysialic acid chains to N-CAM molecules was demonstrated using immunochemistry and two specific probes: endoneuraminidase (endo N) and antiserum H-46, specific for alpha-2,8- polysialic acid chains (Maier et al., 1986). A monoclonal antibody raised against the capsular polysaccharides of the *Meningococcus* group B (anti-Men-B), which shares alpha-2,8 linked sialic acid units in common with PSA-N-CAM (Rougon et al., 1986), specifically distinguishes the highly sialylated (embryonic) from the weakly sialylated (adult) form of N-CAM. This anti-Men-B and site-directed polyclonal antibodies directed against all N-CAM isoforms were used in our immunoblotting and immunohistochemical studies on larval and adult brain and on normal and regenerating SC

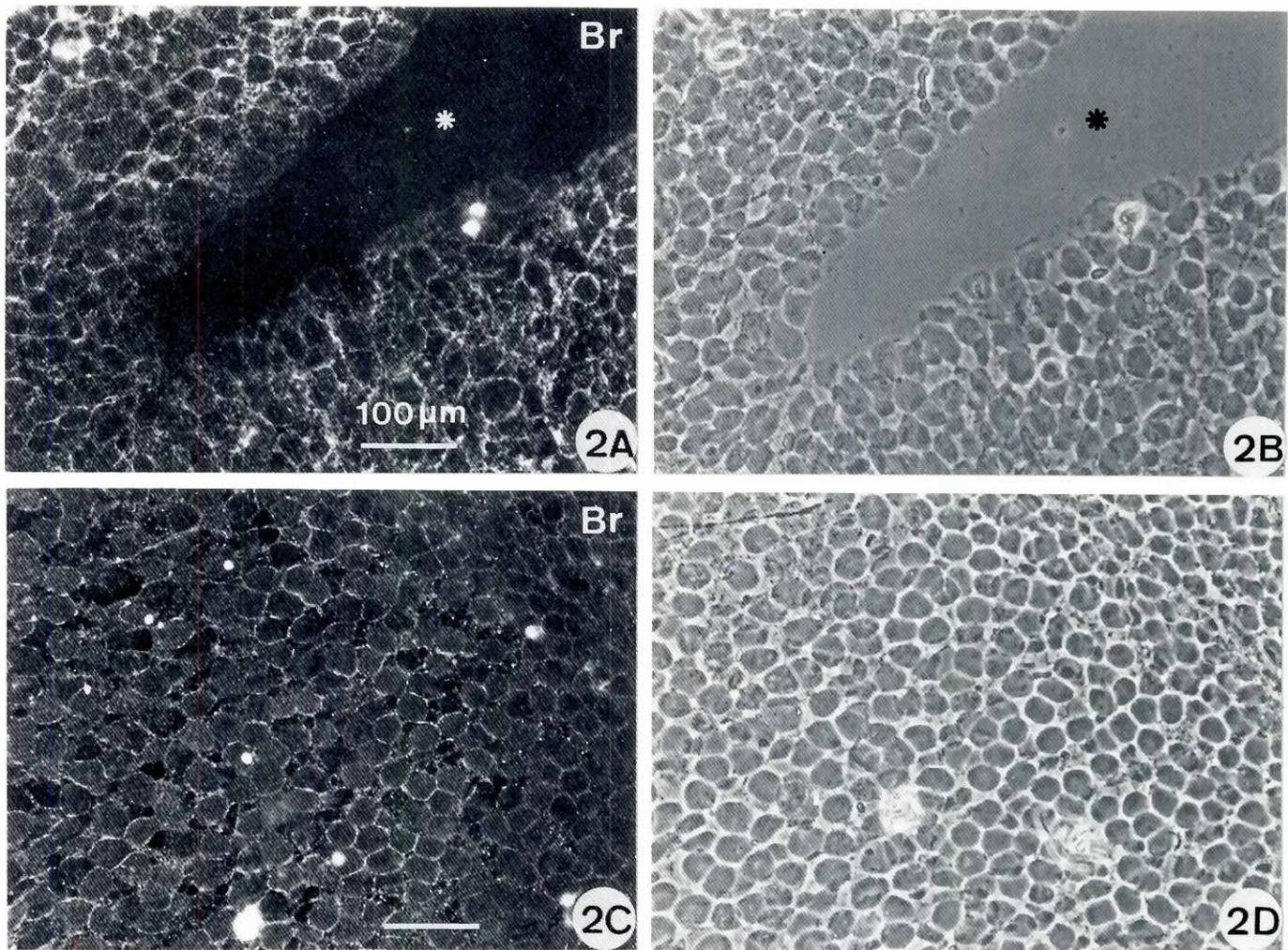


Fig. 2. Parts of sections through grey matter regions of Pw adult brain. **(A)** Total N-CAM localization. Immunoreactivity is seen throughout this CNS area surrounding the ventricular cavity (asterisk). **(B)** Phase-contrast micrograph corresponding to (A). **(C)** PSA-N-CAM labeling. A fine but clear surface reaction is observed around the cells. **(D)** Phase-contrast image of (C).

of adult urodele amphibians. Our results support the hypothesis that, during newt tail regeneration, remodelling processes of ependymal tissue — from which the CS regenerates in adult urodeles (Egar and Singer, 1972) — are correlated with a transient re-expression of PSA-N-CAM in regenerating SC. This high level of PSA-N-CAM immunoreactivity persists throughout the time the ependymal tube is reorganizing the SC cytoarchitecture. Parts of results reported here were presented at two meetings: at the Congress on «Cytoskeleton and Cellular Communications» organized by the «Société de Biologie Cellulaire de France» (Caubit *et al.*, 1991) and at the 11th Annual M. Singer Symposium on «Regeneration and Development» (Caubit *et al.*, 1992).

Results

Western blot analysis

The polysialylated («embryonic») and weakly sialylated («adult») isoforms of N-CAM in samples from CNS of Pw (Fig. 1, lanes 1-3) and tail regenerates of adult Pw (Fig. 1, lanes 4-7) or Nv (Fig. 1, lanes

8-9) were analyzed by immunoblotting with anti-Men-B and anti-total N-CAM antibodies. In developing brain of Pw larvae (lane 1), but also in brain of adult Pw (lane 2), a diffuse material with a range of about 140-220 kDa was stained with anti-total N-CAM polyclonal antibody. In SC extract from adult Pw, two faint bands with apparent relative weight of 140-180 kDa were revealed in a relatively diffuse component by the same total N-CAM polyclonal antibody (lane 3). On the other hand, homogenates from 3 week-old tail regenerates of Pw and Nv were probed using anti-total N-CAM polyclonal and anti-Men-B antibodies without (lanes 4,6,8,9) or after digestion with endo N (lanes 5 and 7). A diffuse staining heterodisperse material was observed with N-CAM polyclonal antibody in blots from tail regenerates of Pw (lane 4) or Nv (lane 8). The protein migration profiles in these blots appeared somehow altered by endo-N treatment (lane 5). However, it is noteworthy that the high degree of N-CAM-associated PSA in regenerating tail extracts from Pw (lane 6) and Nv (lane 9) was more clearly demonstrated by anti-Men-B immunoreactivity, which was completely lost after endo-N digestion (lane 7).

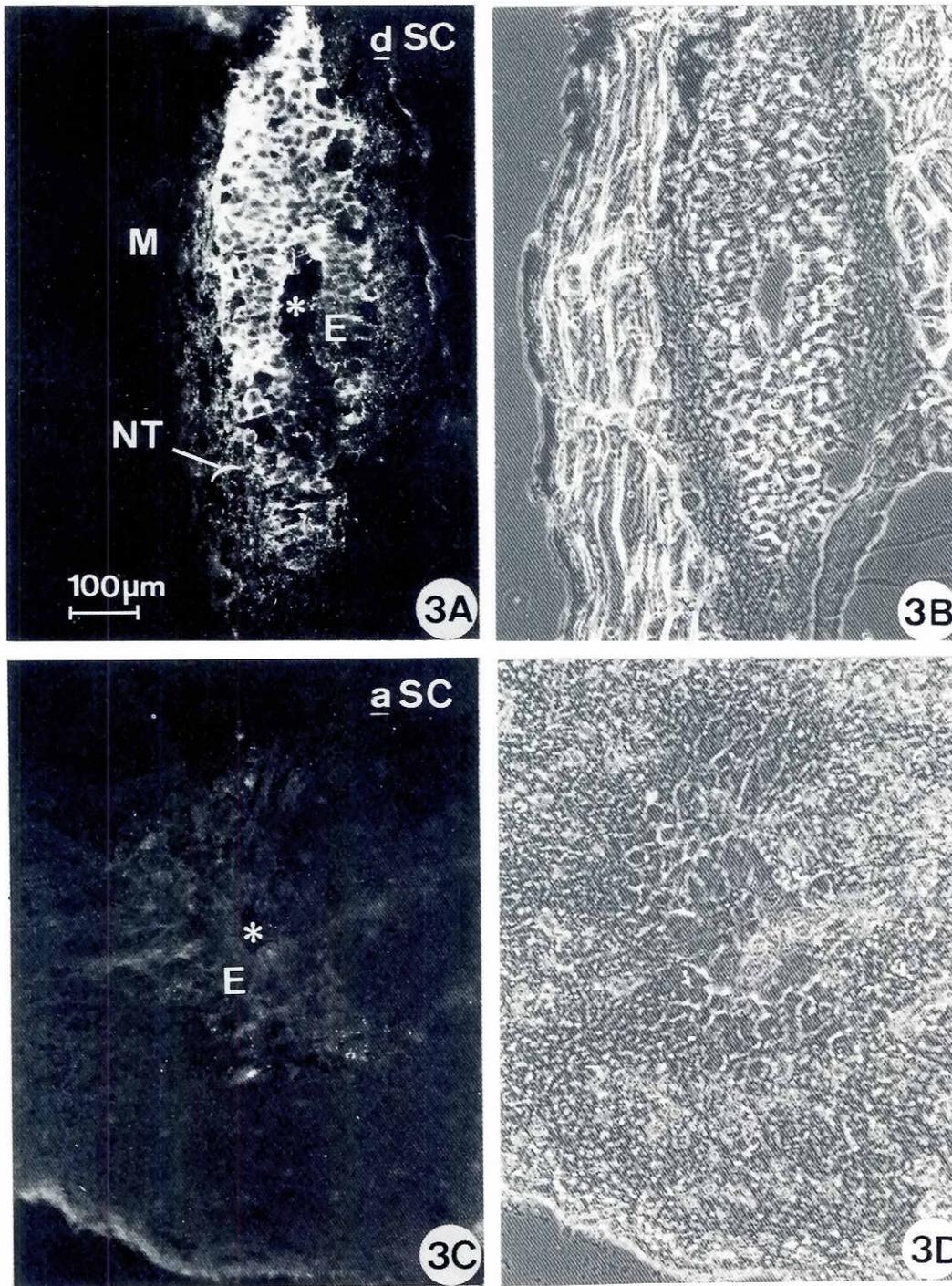


Fig. 3. PSA-N-CAM localization in the SC of larva and adult Pw. (A) Oblique section through developing spinal cord (d SC) of a 3-week-old larva. Intense immunoreactivity is seen at the surface of ependymal cells (E) and in the nervous tractus (NT). M: skeletal muscle. Asterisk: ependymal canal. (B) Phase-contrast image corresponding to (A). (C) Cross-section through adult spinal cord (aSC). A weak immunolabeling essentially restricted to the ependyma (E) is observed. Asterisk: Central canal. (D) Phase-contrast image of (C).

Immunohistochemistry

Localization of PSA and total-N-CAM immunoreactivities in the developing and adult CNS

With anti-total N-CAM polyclonal antibodies, immunostaining appeared widespread throughout the developing or adult brain, e.g. at the surface of ependymal cells surrounding the ventricular cavity (Fig. 2A/B) and on neuronal cell bodies, and axonal tracts in the

white matter (results not shown). With the anti-Men-B antibody, a strong immunoreactivity was observed in the developing brain of larvae, as well as in the brain of post-metamorphic Pw (results not shown). Although the anti-Men-B immunoreactivity was lower in the adult brain of newts, a persistent labeling could be seen in several areas (Fig. 2C/D). Cryostat sections through developing SC in 3-week-old larvae of Pw showed a strong immunoreactivity with anti-Men-B antibody (Fig. 3A/B). The expression of the PSA-N-CAM was

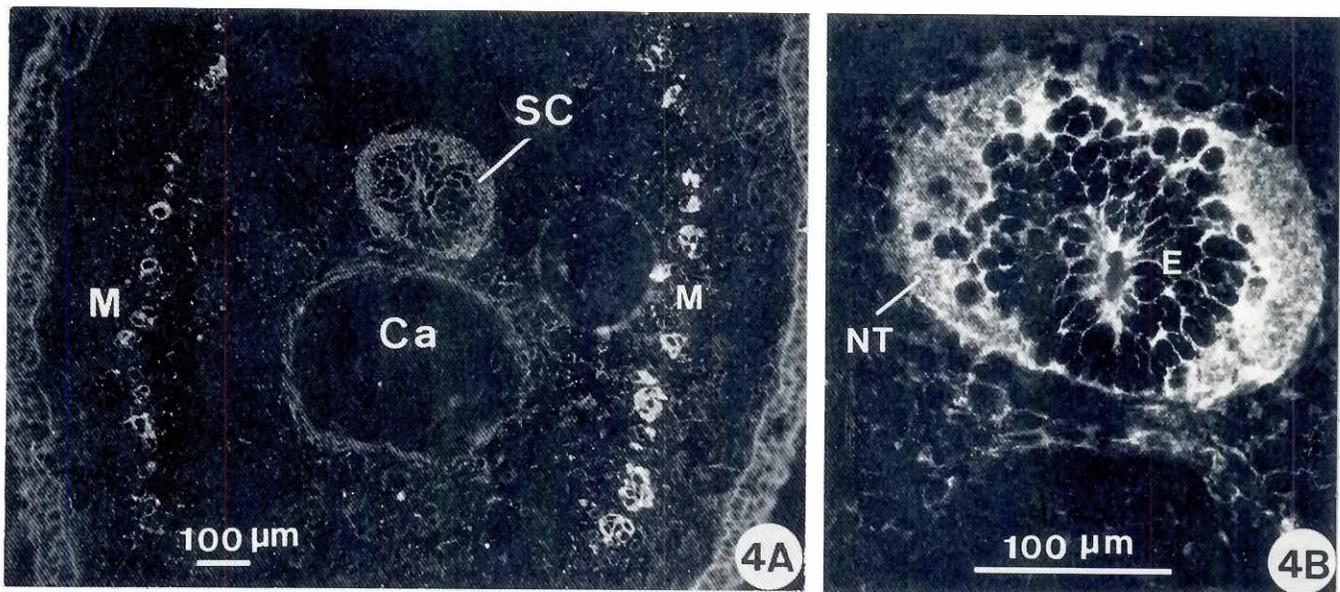


Fig. 4. Total N-CAM localization in cross sections through rostral regions of Nv tail regenerate 2 weeks post-amputation. (A) Immunoreactivity is observed in the regenerating caudal SC, and in the mesenchyme, especially in muscle cell clusters of the premyogenic zones (M). Ca: Cartilaginous vertebral body. (B) Enlarged view of the regenerating SC showing that staining is localized at the cell surfaces of the ependymoglia (E) and in axonal compartments of the nervous tractus (NT). Note also some labeling around the mesenchymal cells.

intense at the surface of the ependymoglia cells and in the nervous tracts (Fig. 3A/B). In the normal adult SC (Fig. 3C/D), PSA-N-CAM was weakly expressed; only the gliopendymal region around the spinal central canal showed some labeling (Fig. 3C/D).

Visualization of PSA and total-N-CAM immunoreactivities during caudal SC regeneration

In order to find out if PSA-N-CAM was re-expressed during morphogenetic events of adult newt tail regeneration, and more especially during remodelling processes associated with the CS regeneration, immunostaining experiments were performed with anti-Men-B and anti-total N-CAM antibodies on transverse and rostro-caudal sections through tail regenerates of Nv or Pw staged as previously indicated (Iten and Bryant, 1976; Khretschatsky *et al.*, 1988; Thouveny *et al.*, 1991). In cross sections through 2-week-old tail regenerates of adult Nv (stage III), a strong marginal staining with anti-total N-CAM antibodies was observed in the reforming SC (Fig. 4A and B). The immunoreactivity was associated with the surface of the ependymoglia cells, and more particularly, the axonal compartments defined by their radial extensions and endfeet processes extending to form the glia limitans (Fig. 4B). Note that expression of all N-CAM isoforms was also observed on the PNS anlagen, i.e. peripheral glia and ventral root sheaths (results not shown). Outside the SC, a weak immunoreactivity was seen in the mesenchyme around the differentiating central cartilage rod and along the epidermal basement membrane (Fig. 4A). Furthermore, cell clusters localized in the premyogenic zone, presumably composed of prefusing myoblasts, were also reactive (Fig. 4A).

Longitudinal sections through stage III regenerating tails of adult Nv revealed an intense and consistent anti-Men-B immunoreactivity all along the ependymoglia cell surfaces and the nervous tracts (Fig. 5A,B,C). Weak or no labeling was observed in the stump region of the SC localized rostral to the plane of amputation (Fig. 5A,D/E),

where numerous processes of histolysis and tissue dedifferentiation occurred. High PSA-N-CAM immunoreactivity was still found in SC by stage IV (i.e. in 3- to 4-week-old regenerates), especially in the caudal part of the SC (Fig. 6A/B), but the expression of this N-CAM isoform decreased then in the regenerated SC to be, by stage V (i.e. 6 to 8 weeks after amputation), as weak (Fig. 6C/D) as it was in the normal adult SC (Fig. 3C/D).

Discussion

In this study, we have examined in amphibian urodeles the patterns of expression of PSA/E-N-CAM and of all N-CAM isoforms during the development of the CNS and the post-traumatic regeneration of the caudal SC. Our main findings may be summarized as follows:

Using the polyclonal anti-total N-CAM antibodies, it has been shown that all N-CAM isoforms are expressed (1) by the neuronal cell bodies and processes, and the ependymoglia cells of the CNS, throughout larval and adult stages, (2) within newt tail regenerates, more especially at the cell surfaces of the ependymal tube and in the axonal compartments, but also, in the mesenchyme of the regenerate.

Using the anti-Men-B antibody, it has been observed that: (1) PSA-N-CAM is strongly expressed in embryonic developing caudal SC, but is weakly expressed in the normal CS of the post-metamorphic and adult newts; (2) there is a striking re-expression of the highly sialylated form of NCAM during CS regeneration following tail amputation. The higher level of expression is seen at the ependymal cell surfaces and in differentiating axonal tracts of reforming SC in 4-to-6 week-old regenerates (stages III and IV). This expression gradually decreases in later stages to nearly disappear in the fully differentiated regenerates (stages V and VI).

Maier *et al.* (1986) demonstrated that N-CAM was involved in

limb regeneration of Nv by using anti-N-CAM Fab fragment which delayed the regenerative process. From this experiment, the authors concluded that limb regeneration was under N-CAM-dependent neuronal influence and/or that N-CAM could play a role in non-neuronal cell-cell interactions in the blastema. Since PSA-N-CAM is thought to control glial and neuronal plasticity, we postulated that this N-CAM isoform could modulate the adhesive properties of blastemal cells during morphogenetic events which take place in amphibian urodeles, in tail as well as limb regeneration, more especially in SC regeneration.

PSA-N-CAM re-expression associated to newt CS plasticity and regeneration

Several lines of evidence suggest that the presence of hydrated polysialyl domain may exert a steric constraint on the protein that regulates the affinity of homophilic binding of cell adhesion molecules. The presence of PSA has been suspected to affect not only cell-cell adhesion but also cell-substrate adhesion as demonstrated *in vitro* by Acheson *et al.* (1991). In addition to regulation of the affinity of N-CAM homophilic binding, extended polysialic acid polymers of N-CAM can exert a more general regulation of cell membrane apposition (Rutishauser *et al.*, 1985). This raises the possibility that retention or re-expression of these embryonic features could be associated with neuroplasticity in adulthood. Two non-exclusive possibilities emerge: (1) PSA-N-CAM could be maintained throughout adult life in well-defined CNS regions which undergo structural changes. (2) PSA-N-CAM could be re-expressed in certain CNS areas following injury or disease.

High level of PSA-N-CAM coincides with the occurrence of remodelling processes taking place in several centers of the brain in adult mammals. This is true, in particular, of the olfactory bulb (Miragall *et al.*, 1988), the substantia nigra (Aaron and Chesselet, 1989), the hippocampus (Goldowitz *et al.*, 1990) and the hypothalamo-neurohypophysial system (Theodosis *et al.*, 1991). In all these CNS areas, plasticity and cell reshaping are correlated with the maintenance of PSA-N-CAM expression in the adult tissues. Using the anti-Men-B antibody to map immunohistochemically and in detail the distribution of PSA-N-CAM throughout the CNS of the adult rat, Bonfanti *et al.* (1992) have recently found immunoreactivity for this N-CAM isoform in several centers of the adult brain, including areas which are not yet known to undergo structural reorganization.

In glial cells of rat hippocampus in which status epilepticus induced by kainic acid (Le Gal-La Salle *et al.*, 1991), highly polysialylated form of N-CAM was re-expressed. The possibilities of re-expression of «embryonic» form of N-CAM at the lesion site, such as in the latter case, may be a prerequisite for brain damage repair. This *in vivo* description of a reconversion towards PSA/E-N-CAM expression in the CNS supported the hypothesis that immature features present during development could be sometimes re-established in pathological or traumatic situations.

During tail regeneration of urodele amphibians, while all the

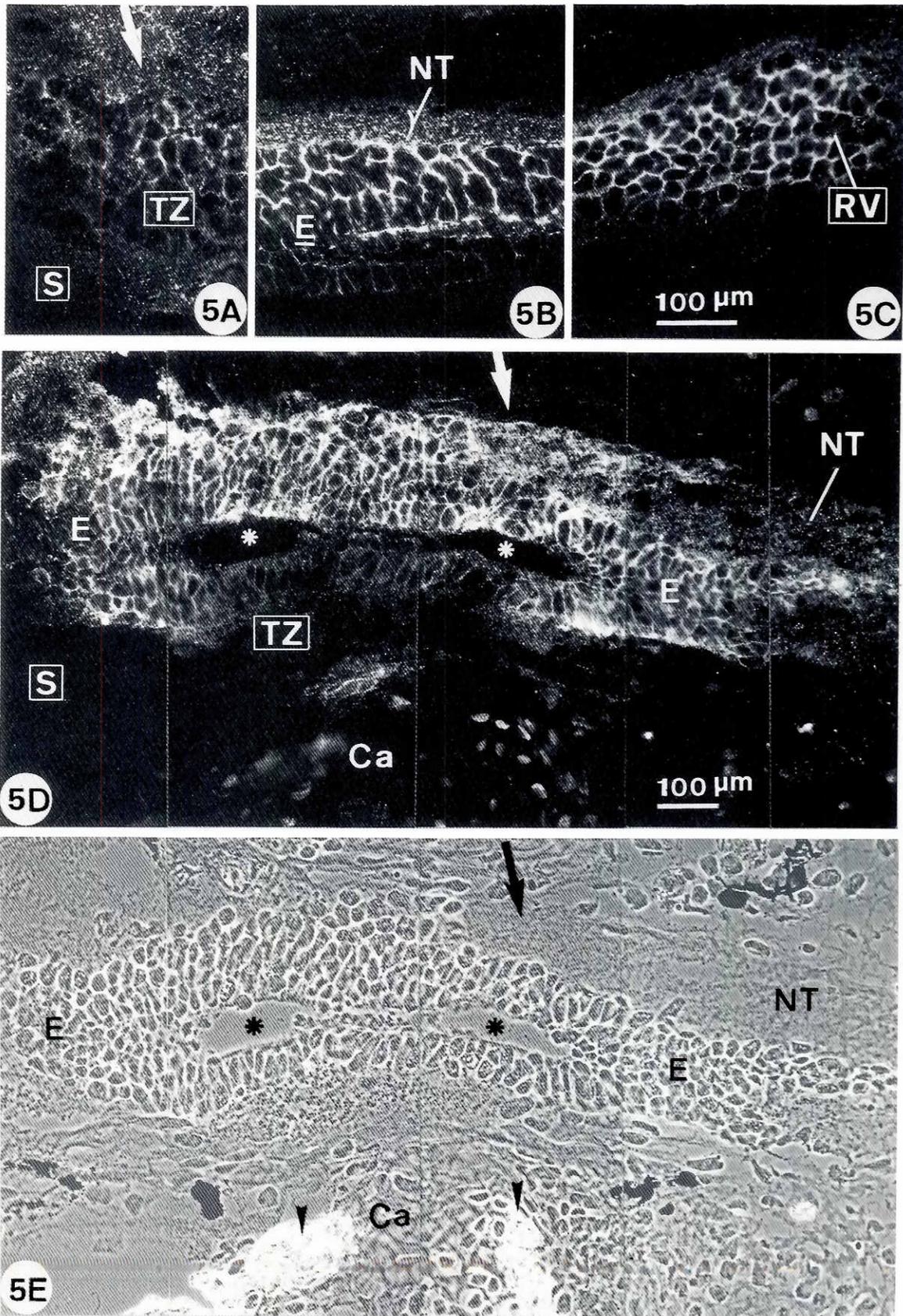
structures are progressively re-established within the regenerate, the regenerated ependymal tube acts as a primordial axial structure which allows the regeneration of the CNS (spinal cord) and the PNS (ventral roots and spinal ganglia) and induces the differentiation of the cartilaginous rod and segmented muscles (Holtzer, 1956). The characteristic re-expression of PSA-N-CAM, at the level of SC amputation, is associated with the remodelling processes of the neural tissue, allowing the formation of a contingent of neural cells able to divide, to migrate and to reconstitute the caudal CNS. At this level, the re-expression of «embryonic» form of N-CAM seems to reproduce what is happening during larval SC development, when the CNS growth is more effective. Since it has been proposed that immature but not adult glial cells can promote growth during regeneration processes (Smith *et al.*, 1990), the ependymal cells, which still express PSA-N-CAM at the amputation level, could produce the cells involved in SC regeneration. However, it seems that their number is not sufficient to give rise to all the cells participating in the ependymal tube formation, in spite of their mitotic proliferation activity. Therefore, one could also propose a reversion (dedifferentiation) process of the mature glial cell type to an immature one which can re-express PSA-N-CAM and divide. During the migration of cells into the regenerate, the presence of PSA-N-CAM at the cell surface could regulate both cell-cell and cell-substrate interactions, as demonstrated *in vitro* by Acheson *et al.* (1991).

PSA-N-CAM expression and neurite outgrowth

The high amount of PSA on N-CAM in the axonal compartments of the regenerating as well as developing SC in newts and the loss of PSA in the adult CS, is consistent with the view that PSA modulates the neurite outgrowth-promoting activity of N-CAM. This idea is supported by *in vitro* studies which have shown that removal by endo-N of neuronal PSA from the rat cerebellar granule cells inhibits by 70-80% the N-CAM component of neurite outgrowth on N-CAM 140-transfected cells (Doherty *et al.*, 1990, 1992). According to Doherty *et al.* (1992), although the molecular basis of PSA modulatory effects on N-CAM-dependent neurite outgrowth remains to date unknown, PSA appears as an important determinant of the ability of neuronal N-CAM to act as a receptor that transduces the homophilic binding signal into a cellular response. PSA may be considered as a positive modulator of neurite outgrowth-promoting activity of N-CAM (Doherty *et al.*, 1990), whereas it acts as a negative modulator of adhesion (see e.g. Hoffman and Edelman, 1983; Boisseau *et al.*, 1991; Bonfanti *et al.*, 1992). The high level of expression of PSA on embryonic axons, at the time of their extension, could allow them to avoid adhesion processes such as the establishment of stable interactions during fasciculation while retaining the capacity to recognize relevant guidance and target cues (Jessel *et al.*, 1990; Acheson *et al.*, 1991).

In conclusion, immunohistochemical evidence was obtained that showed that capacity of SC plasticity during SC regeneration in adult newts was associated with a transient increase of PSA-N-CAM

Fig. 5. PSA-N-CAM localization in regenerating caudal SC. (A-C) PSA-N-CAM immunoreactivity at the proximal (A), median (B) and distal (C) levels of a longitudinal section through regenerating SC of a 2-week-old Nv tail regenerate. Only the regenerating part of the SC is brightly stained, the stump (S) being weakly or not labeled, except the transition zone (TZ) close to the amputation plane (arrow). At the different levels of the regenerating SC, a strong surface reaction is seen over the ependymoglia (E), and a reaction is clearly observed along the nervous tracts (NT). RV: distal regeneration vesicle. **(D)** PSA-N-CAM immunolabeling in oblique section through the stump transition zone (TZ) of a 2-week-old regenerating caudal SC of Nv. Staining is seen at the surface of ependymoglia cells (E) and in the nervous tract (NT). Asterisks: ependymal canal. Ca: cartilage. Arrow: approximative level of the amputation plane. **(E)** Phase-contrast image of (D). Note that cartilage (Ca) is appearing at the end of the lesioned vertebral bone (arrowheads).



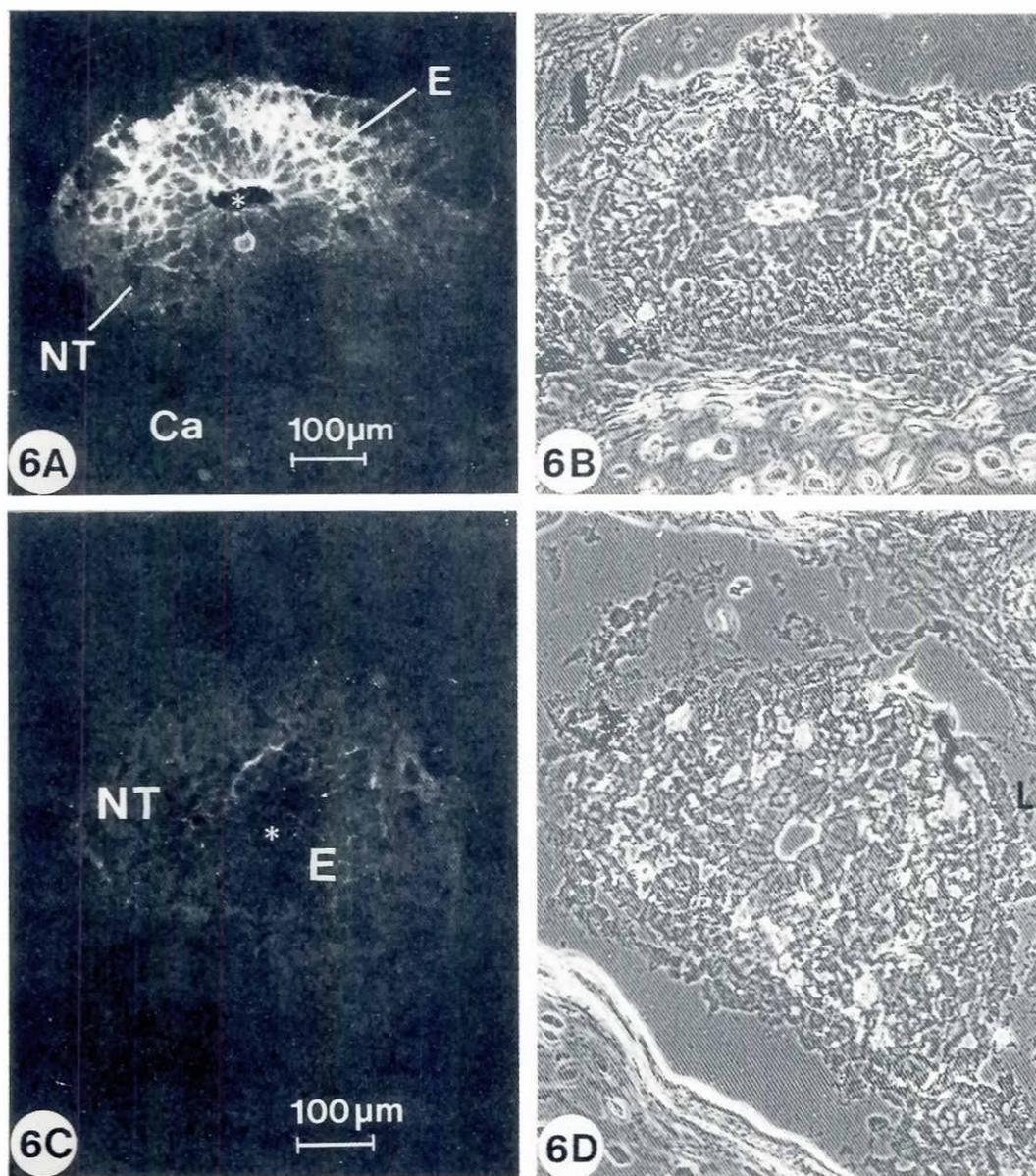


Fig. 6. PSA-N-CAM immunoreactivity in cross-sections through regenerating caudal SC of adult Pw. (A) 4-week-old regenerating SC. Intense labeling is observed at the surface of ependymoglial cells (E) and in the nervous tracts (NT). Ca: cartilaginous vertebral body. Asterisk: ependymal central canal. (B) Phase-contrast micrograph of (A). (C) 8-week-old regenerating SC. Immunostaining is weak at this regeneration stage in ependyma (E) as well as in the nervous tractus (NT). Note however the presence of reactive sites around some ependymal cells. (D) Phase-contrast image of (C).

at the surface of the ependymoglial cells whereas the properties of structural remodelling of certain adult brain regions in mammals is correlated with a retention of a high expression level of this N-CAM isoform. In addition to its determinant role in morphogenetic events of the regenerating ependymal tube, PSA-N-CAM almost certainly influences nerve growth and sprouting during the reorganization of the SC axonal network. Finally, because the mesenchymal cells of the blastema also showed immunoreactivity, with anti-total N-CAM and anti-Men-B antibodies, some more general role for N-CAM could be postulated in cell-cell and cell-substrate interactions during the formation of the regeneration blastema.

Materials and Methods

Animal surgical procedures

The urodele amphibians used in this study were *Pleurodeles waltl* larvae

and adult newts. *Pleurodeles waltl* (Pw) were obtained from the C.N.R.S. Amphibian Farm, Centre de Biologie du Développement, Université Paul Sabatier, Toulouse, France; *Notophthalmus viridescens* (Nv), were obtained from Lee's Newt Farm, Oak Ridge, Tennessee and from Nasco, Fort Atkinson, Wisconsin. Animals were reared in groups of 10-12 and maintained in circulating tap water thermostated at 18-20°C; the water was completely renewed twice a week. Pw were fed twice a week with beef heart or liver, and Nv with tubifex. Pw larvae were obtained from egg layings in the Laboratory.

Before surgery, adult animals were anesthetized with 1:1000 MS 222 (tricaine methane sulfonate, Sigma). Amputations were performed in the third rostral part of the tail, at the level of an intervertebral junction. Operated animals were observed daily and staged according to Iten and Bryant (1976) and Thouveny *et al.* (1991) for Nv, and to Khretschatskiy *et al.* (1988) for Pw.

After appropriate periods of regeneration, the blastema were harvested by reamputation. Samples covering the five stages of tail regeneration were used in this study.

Antibodies

Two types of antibodies were used :

(1) A mouse monoclonal immunoglobulin M (IgM), raised against the capsular polysaccharides of *Meningococcus* group B (anti-Men-B), that shares alpha-2,8-linked N-acetylneuraminic (polysialic) acid residues: this antibody specifically recognizes the highly polysialylated («embryonic») form of N-CAM (Rougon *et al.*, 1986).

(2) A site-directed rabbit polyclonal serum that recognizes the NH₂-terminal residues of N-CAM sequence which is shared by all isoforms (Rougon and Marshak, 1986) and an anti-chicken total N-CAM polyclonal antibody (gift of Dr. J.P. Thiery).

SDS/PAGE and Immunoblot analysis

Cross-reactivity of antibodies with urodele amphibian antigens was controlled by Western blotting. Immediately after dissection, samples (brain, SC and tail regenerates) were homogenized in 7 volumes of 20 mM Tris-HCl, 10 mM EDTA, 0,2% Triton x100, 1 mM Phenylmethyl sulfonyl fluoride (PMSF), 1 µg/ml antipain and pepstatin, 15 µg/ml benzamidine pH 8 (antipain, pepstatin and benzamidine were first solubilized in DMSO). The homogenization was carried out on ice in a Dounce homogenizer with a glass pestle for brain. Before homogenization, regenerates were crushed in an Ultra-Turax T25 tissue grinder for 15-20s. Samples were then sonicated and centrifuged at 10,000g for 10 min at 4°C. Proteins present in the supernatant were resolved by 7% SDS PAGE (Laemmli, 1970). Proteins were then transferred onto nitrocellulose membrane according to Burnette (1981).

Radioimmunodetection of N-CAM

Nitrocellulose sheets were soaked in saturation buffer (3% low-fat milk in PBS) for 1 h at room temperature and incubated overnight at 4°C with anti-Men-B antibody used at a dilution of 1:500 in the same buffer. After washes, the sheets were reacted for 4 h at room temperature with rabbit anti-mouse IgM antibody (1:200 dilution) as secondary antibody. Bound antibodies were detected by incubation for 35 min at room temperature with ¹²⁵I protein A (0.5x10⁶ CPM/ml). The dried nitrocellulose sheets were autoradiographed with Fuji films. Polyclonal anti-total NCAM antibody was directly revealed with ¹²⁵I-protein A.

In order to check the presence of N-CAM-associated PSA in diffuse stained components of immunoblots, some samples were treated before the electrophoretic migration with a viral endoneuraminidase (endo N) prepared according to Boisseau *et al.* (1991). Tissue extracts were incubated for 4 h at 37°C with this bacteriophage endosialidase (activity: 3.5x10¹¹ pfu ml⁻¹), which is known to remove alpha-2,8-linked PSA chains (Vimr *et al.*, 1984).

Indirect immunofluorescence

Samples were directly embedded unfixed in OCT and frozen in liquid nitrogen. Longitudinal and cross sections of 15 µm were cut in a cryostat at -22°C. They were collected on gelatinized slides and stored at -20°C. They were washed 1 h in PBS+1% BSA, then incubated 1 h with primary antibodies used at a 1:50 dilution for anti-Men.B and anti-total-N-CAM antibodies. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM or anti-rabbit IgG were used at a 1:100 or 1:200 as secondary antibodies. Washed slides were mounted in moviol and observed with epifluorescence Zeiss microscope and photographed on Tri-X pan (Kodak). Controls were made by omitting the first antibody or by replacing it with preimmune serum.

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