Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8395–8399, November 1986
Neurobiology

Expression and function of neural cell adhesion molecule during limb regeneration

(cell–cell adhesion/axon–mesenchyme interaction/nerve-dependent limb regrowth)

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Communicated by Lester O. Krampitz, July 7, 1986

ABSTRACT **The neural cell adhesion molecule (NCAM) has been detected in regenerating limb bud of adult newts in addition to brain and peripheral nerves. In the regenerating tissue, NCAM was found primarily on mesenchymal cells and also in wound epidermis. Infusion of Fab fragments of antibodies to NCAM into limb buds at the early blastema stage delayed the regenerative process. Previous studies have indicated that NCAM serves as a homophilic ligand for adhesion among cells that express this molecule and, in doing so, can influence the interaction of nerves with their environment. The expression of NCAM in regenerating limb and the effects of antibody infusion are therefore consistent with the observation that limb regeneration requires interactions among axons and mesenchymal cells.**

Urodele amphibians possess the ability to regenerate amputated limbs, and it has been known since the original studies of Todd in 1823 (1) that this phenomenon is dependent on the presence of nerve fibers. A newt regenerates a new limb in three phases: (*i*) wound healing—a closing of the wound by the wound epidermis and the phagocytic removal of the debris caused by amputation; (*ii*) accumulation—an increase in the number of undifferentiated, mesenchyme-like cells beneath the wound epidermis to form the regeneration blastema; and (*iii*) differentiation—a process that begins when a critical mass of mesenchymal cells has been reached and ends with a regenerated limb that is frequently indistinguishable from that which it replaced (2). Though all phases of newt limb regeneration are nerve dependent, the accumulation phase is most dramatically affected by nerve withdrawal. If a limb is denervated just before or at the time of amputation, wound healing proceeds but no limb bud is formed. When a limb is denervated during the accumulation phase, mitotic activity ceases, with the result that the limb bud withers and is resorbed. Denervation during the differentiation phase halts mitosis, but the cells existing at the time of denervation continue to differentiate to form a miniature limb (2). The quantitative dependence of limb regeneration on a neural influence has been shown with more precision in studies using partial denervations. The newt forelimb, which contains more than the minimum threshold number of axons required to support limb regeneration (2), is innervated by brachiospinal nerves 3–5, each of which contributes differing axon quantities to the motor and sensory innervation of the limb. When various combinations of the three nerves are cut at the time of amputation, the resulting delays in the onset and the slowing of the rate of limb regeneration are in direct proportion to the remaining number of axons (3, 4). Employing a nerve conditioning lesion (5) or supplying denervated limb buds with brain homogenates (6) further demonstrates the trophic effect of neurons on limb regeneration. The neurotrophic effect is probably mediated by a membrane-bound factor(s) that is axonally transported with the fast component (7).

After a newt limb is amputated, all of the tissues of the limb bud are formed by differentiation of the mesenchymal cells of the regeneration blastema—all tissues, that is, except the axons cut at the time of amputation. The new (daughter) axons are the result of direct outgrowth from preexisting (parent) axon stumps, and it is the newly formed daughter axons that convey the neurotrophic factor(s) for limb regeneration and ultimately innervate the new limb.

The neural cell adhesion molecule (NCAM) has been shown to function as a homophilic ligand in the interaction of neurons with a variety of other cell types, including muscle, glia, and other neurons (for review, see refs. 8 and 9). As in the case of neurotrophic factor(s), NCAM has been reported to be axonally transported in the fast component (10). It has been proposed that NCAM can serve as an adhesive substrate for guidance of axon growth cones through a terrain containing cells that also express this molecule. For example, NCAM has been found to be localized on the marginal endfeet of radial neuroepithelial cells, and the presence of antibodies against NCAM can alter the stereotyped route of retinal ganglion cell axons to the optic tectum (11). Given these observations, and the fact that limb regeneration is dependent on the regrowth of neurons into the limb, we have carried out studies designed to determine whether NCAM is present in the regenerating limb bud and, if so, whether antibodies to NCAM would have an effect on limb regeneration.

MATERIALS AND METHODS

Identification of NCAM in Newt Tissues. Newt (*Notophthalamus viridescens*) brain, mature limb, regenerating limb bud, or brachiospinal nerve tissues were dissected on the day of sample preparation for sodium dodecyl sulfate/polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE) (12). All subsequent procedures were carried out on ice. Tissues were pelleted and sonicated in phosphate-buffered saline (PBS) and in some cases treated with a viral endoneuraminidase [endoneuraminidase (endo N)] for 15 min [20 units/mg of protein, generously supplied by E. Vimr and characterized according to Vimr *et al.* (13)]. All sonicates were diluted 1:2 with 2× concentrated sample buffer (0.0625 M Tris·HCl buffer, pH 6.8/2% $NaDodSO_4$/5% 2-mercaptoethanol/10% glycerol/0.001% bromphenol blue) and boiled for 3 min. Proteins were separated on 7% polyacrylamide gels by Na-

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Abbreviations: NCAM, neural cell adhesion molecule: PAD, postamputation day; endo N, endoneuraminidase; successive stages of regeneration: ME, moderate early bud; E, early bud; AE, advanced early bud; MB, medium bud; LB, late bud; Pal, palette.
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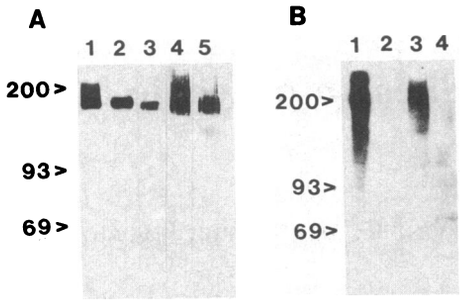

FIG. 1. Characterization of newt NCAM by NaDodSO$_4$/PAGE and immunoblots using antibodies to frog NCAM. (*A*) Adult newt brain NCAM (lane 4) is similar to adult frog brain NCAM (lane 1). The NCAMs appear as a faint band at 140 kDa, a dark band at 180 kDa, and a diffuse staining extending from the distinct bands to 250 kDa. Endo N treatment, which removes NCAM-associated polysialic acid, reveals discrete components of frog brain NCAM (lane 2) and newt brain NCAM (lane 5) that are similar to those found in endo N-treated embryonic chicken brain (lane 3). (*B*) Polysialylated material with a range of 140–250 kDa was recognized by antiserum H.46 in embryonic chicken (lane 1) and adult newt (lane 3) brain extracts. Endo N eliminated H.46 staining in chicken (lane 2) and newt (lane 4) brain extracts. Molecular mass standards were ^{14}C-radiolabeled myosin (200 kDa), phosphorylase B (93 kDa), and bovine serum albumin (69 kDa).

DodSO$_4$/PAGE and transferred to nitrocellulose filters (Schleicher & Schuell, 0.45 μm) by electrophoresis (14). NCAM was detected on the nitrocellulose filters by using a modified (15) indirect immunostaining procedure (14). The filters were incubated either with purified IgG from rabbits immunized with affinity-purified frog brain NCAM (anti-NCAM) or normal rabbit IgG, washed, and then treated with peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). The bound peroxidase was detected by using the chromogenic substrate 4-chloro-1-naphthol (16). For detection of α-2,8-linked polysialic chains, H.46 antiserum [from the laboratory of J. B. Robbins and characterized by Finne and Makela (17) and Vimr *et al.* (13)] was used as the first antibody followed by rabbit anti-horse IgM antiserum (Cappel Laboratories) as the second antibody and ^{125}I-labeled goat anti-rabbit IgG (New England Nuclear) as the third antibody. Bound radioactivity was detected by using Kodak X-Omat AR x-ray film with an enhancing screen (18).

Staging of Limb Regeneration. In all morphology and histology studies, adult newts were bilaterally amputated through the distal third of the humerus while under tricaine methanesulfonate anesthesia. When limb growth was detected in the early accumulation phase, the limbs either were infused with anti-NCAM Fab (or control substances) and

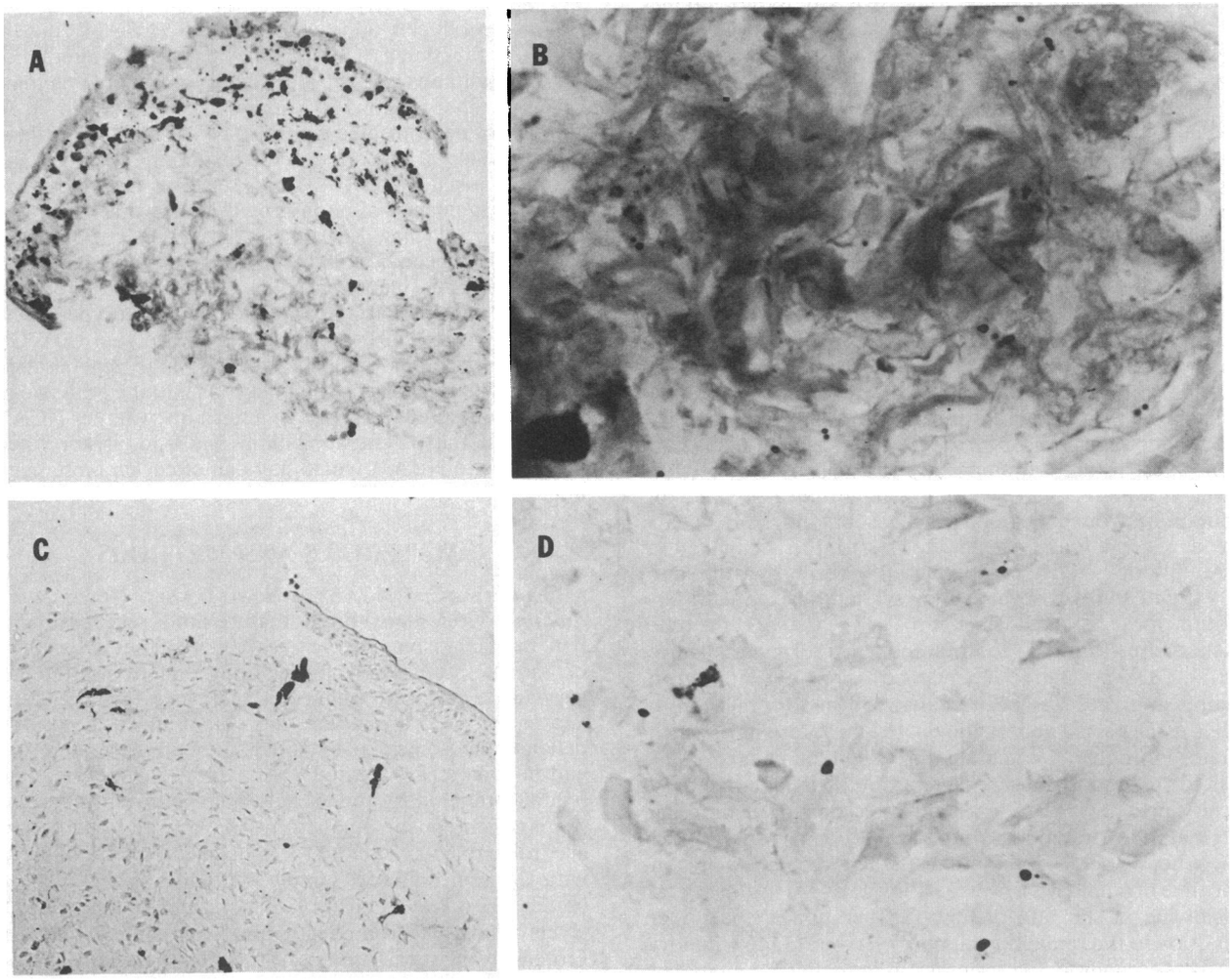

FIG. 2. Sections of ME–E-stage limb buds exposed to anti-NCAM IgG (*A* and *B*) or nonimmune rabbit IgG (*C* and *D*) and stained by using peroxidase-conjugated anti-rabbit IgG. There is no reaction product on the cells exposed to nonimmune IgG (*C* and *D*) but a light grey hue allows these unstained cells to be photographed. The brown diaminobenzidine reaction product and the light grey background are seen on the mesenchymal cells in *A* and at a higher magnification of the same section in *B*. Antigenic determinants for NCAM are present on cells of the wound epidermis (*B*). Pigment granules of equal intensity are present in the experimental and control limb buds. (*A* and *C*, ×55; *B* and *D*, ×410.)

observed daily to assess the rate of regeneration or were fixed for histological processing, as described below.

The progress of limb regeneration was determined by using an established staging system that has been described in detail (2, 19). Briefly, the stages are *moderate early bud* (ME), a pie-shaped growth characterized by fluidity; *early bud* (E), a dome-shaped growth; *advanced early bud* (AE), a pointed dome; *medium bud* (MB), a cone-shaped growth; *late bud* (LB), an elongated cone; and *palette* (Pal), an elongated cone with distal flattening.

Infusion with Antibody. Animals to be infused were wrapped in moist cotton and strapped to a 2.5 × 7.6 cm glass slide with rubber bands. A glass needle, attached to an infusion pump by tubing, was inserted into the ventral base of the limb and worked distally into a left ME- or ME–E-stage limb bud. A volume of 7.0 μl of infusate was delivered over a 0.5-hr period. After infusion the animals were returned to aquaria. Three different series of animals received infusions of Fab fragments (5 mg/ml) prepared from anti-NCAM IgG. In the first series, the left limbs of 4 animals were infused with Fab on postamputation day (PAD) 16 and the infused left and uninfused right limbs were fixed for histology on PAD 21. There was no daily staging of growth in this pilot series. In the second series, the left and right limbs of 7 animals were infused on PAD 15 when all limb buds were at ME or ME–E stages of growth: the left limb was infused with anti-NCAM Fab and the right was infused with Fab from antibody of unimmunized rabbits. Daily staging of regenerative growth was carried out until both limbs had reached the Pal stage. In the third series, three groups of animals had only left limb buds infused at ME or ME–E. The first group (n = 11) was infused with anti-NCAM Fab and the second (n = 8) was infused with nonimmune rabbit Fab. The third group (n = 7) was not actually infused but simply had the needle inserted into their limb buds. Limb buds were staged for regrowth until both limbs had reached Pal.

Histology. To determine the presence of NCAM in limb buds at the ME, ME–E, or E stage of regeneration, the tissue was fixed for 3–12 hr in 4% paraformaldehyde/7% sucrose made up in phosphate buffer (PBS) at pH 7.4. The limbs were washed in 1 M sucrose/PBS for 3–12 hr and then sectioned at 8 μm on a Slee cryostat. The sections were exposed to the first antibody (rabbit anti-NCAM) for 30 min–1 hr and washed three times for 15 min each in PBS. The sections were then exposed for 30 min–1 hr to the second antibody (goat anti-rabbit IgG) conjugated to horseradish peroxidase, washed three times for 15 min each in PBS, and allowed to react with diaminobenzidine at 50 μg/ml in Tris buffer (pH 7.4) containing 0.3% H_2O_2. After washing, the sections were mounted and viewed by bright-field microscopy.

The tissue structure of the limbs was assessed in four animals, each of which had the left limb bud infused with anti-NCAM at 16 days after bilateral amputation. At PAD 21, the anti-NCAM-infused left limb and the contralateral, noninfused, control right limb of each animal were fixed in Bouins' solution, embedded in paraffin, cross-sectioned at 8 μm, stained with the Bodian (20) nerve stain, and counterstained with Orange G. The number of axons was counted in sections 250 μm distal and 750 μm proximal to the amputation level of the left limbs (21).

RESULTS

Characterization of Newt NCAM. The presence and form of NCAM in newt tissues was assessed by anti-NCAM immunoblots of proteins separated by $NaDodSO_4$/PAGE. The specificity of the rabbit anti-frog NCAM antibody used in these studies was indicated by the fact that they stained only one of the many membrane proteins present in the $NaDodSO_4$/polyacrylamide gels of total brain membranes.

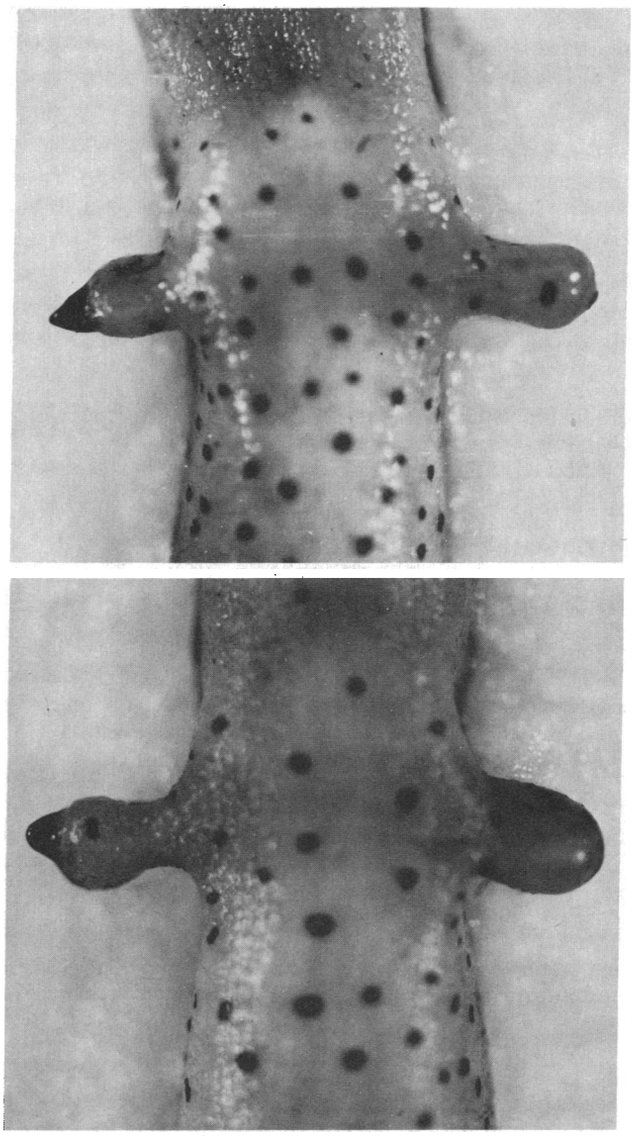

FIG. 3. Effect of anti-NCAM Fab on limb regeneration. Left limb buds were infused with 7 μl of Fab at 15 days after bilateral amputation. Photographs, showing a ventral view, were taken at PAD 21. The anti-NCAM-infused left limb buds of both animals remained at ME while the noninfused right limb bud of the animal in *Upper* grew to Pal and that of the animal in *Lower* grew to between LB and Pal. (×5.)

The stained component was identical in electrophoretic mobility to that reported (22) for NCAM from other species—that is, diffuse stain extending from about 140 kDa to >250 kDa (Fig. 1*A*). Similar components were detected in peripheral nerve, limb, and regenerating limb bud. However, the bands obtained were too faint for photographic reproduction, even with tissue pooled from 38 animals.

The antigen detected in these immunoblots contained α-2,8-linked polysialic acid chains, which are characteristic of NCAM (22, 23). The presence of this sugar moiety was demonstrated by the use of two specific probes, the enzyme endo N (9, 13) and antiserum H.46 (24). Endo N, which cleaves α-2,8-linked polysialic acid chains that are eight sialic acids in length or longer (17) altered the $NaDodSO_4$/PAGE profile of the material stained by anti-NCAM in newt and frog brain (Fig. 1*A*). H.46 antiserum, which is also specific for α-2,8-linked sialic acid chains longer than eight sialic acids in length, stained glycoproteins from chicken and newt brain (Fig. 1*B*). With both species, the H.46-reactive material had

a diffuse electrophoretic pattern and was destroyed by treatment with endo N (Fig. 1*B*).

NCAM Localization in Limb Buds. Microscopy of sections exposed to anti-NCAM and stained by immunoperoxidase methods revealed that NCAM antigenic determinants are present on mesenchymal cells and the wound epidermis of early accumulation-phase limb buds (Fig. 2 *A* and *B*). These methods did not allow visualization of regrowing limb bud axons, which, in our experience, can only be identified with certainty at the light microscopic level by employing nerve-specific stains. Based on the distribution of Bodian-stained axons and the immunoperoxidase stain for NCAM, there is little doubt that limb bud axons are in close proximity to the NCAM-positive mesenchymal cells. Control sections stained using Fab from unimmunized rabbits showed no reaction product in wound epidermis or on mesenchymal cells (Fig. 2 *C* and *D*).

Effect of Anti-NCAM on Limb Growth. These studies were carried out in three independent experiments with a total of 37 animals. In the initial series, infusion of anti-NCAM at PAD 16 (ME stage) delayed regrowth over the next 5 days in three of four limbs. The antibody-injected limbs remained at the same stage of regeneration attained at the time of infusion—i.e., ME—while the uninfused, control limbs had grown substantially to either Pal, LB–Pal, or MB (Fig. 3). Both limbs of the fourth animal remained at ME.

A second series of seven animals had both forelimbs infused, the left with anti-NCAM Fab and the right with Fab from unimmunized rabbits. Growth was recorded daily until both limbs completed Pal stage. In three of the seven animals, the anti-NCAM-infused limb lagged two or more stages behind the contralateral control. Both limbs of the other four animals had equivalent growth rates. We suggest that the smaller number of limb buds affected by anti-NCAM in this series reflects the degree of physical handling required for bilateral infusion. Immediately after infusing anti-NCAM, the animals were unwrapped and then rewrapped in moist cotton with the control limbs exposed for subsequent infusion and the just-infused experimental limb encased within the wrapping. Finally, the animals were restrapped to glass slides by rubber bands. Such treatment may have abraded the gelatinous limb bud or milked the limbs, causing a loss of the recently infused anti-NCAM solution.

Left limbs only were infused in the third series. In the anti-NCAM group, 8 of 11 infused limbs were affected compared to contralateral uninjected controls. Growth was equivalent on both sides of all eight animals infused with nonimmune Fab. Six of seven animals in which an empty infusion needle was inserted into a left limb bud showed equal growth rates in both limbs; the left limb of the seventh animal in this group grew more slowly than the right. The effect of anti-NCAM Fab, normal rabbit Fab, and needle insertion on the rate of limb regeneration compared to contralateral controls is depicted in Fig. 4.

Effect of Anti-NCAM on the Number of Limb Bud Axons. In the initial anti-NCAM infusion experiment, axons were counted in the infused and contralateral limbs of the three affected animals. As seen in Table 1, the number of axons at 750 μm proximal to the amputation level is similar in normal (amputated only) limbs at ME, anti-NCAM-infused limbs at ME, and anti-NCAM contralateral (amputated only) limbs at advanced stages of regrowth. This indicates that the number of axons proximal to the amputation was not affected by either the operation or the antibody infusions. At 250 μm distal to the amputation level, the number of axons in normal limb buds at ME and anti-NCAM-infused limb buds at ME was essentially the same. However, the contralateral control limb buds, whose growth was not delayed by anti-NCAM, had twice the number of axons. Thus, in the experimental and control limbs, the stage of regeneration correlated with the number of axons distal to the amputation, not the time elapsed after amputation.

DISCUSSION

NCAM has been found on the surfaces of neurons, glia, and muscle cells and shown to participate in a variety of interactions among these cell types (8, 9). The present results further expand the range of NCAM-mediated phenomena by demonstrating that the molecule is present on mesenchymal and epidermal cells during the accumulation phase of regenerating limb buds and that the infusion of the limb bud with anti-NCAM Fab retards the regenerative process.

The observation that antibodies to NCAM slow, but do not stop, limb regeneration suggests that a transient or partial effect is produced by a single infusion of anti-NCAM or that

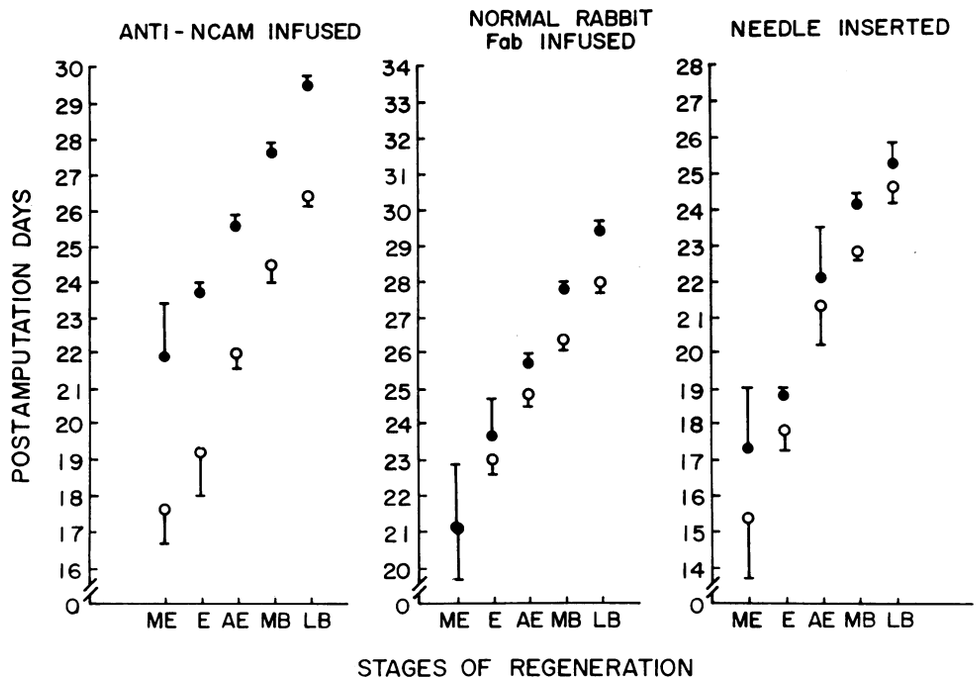

FIG. 4. Effect of anti-NCAM Fab infusion, normal rabbit Fab infusion, or insertion of an infusion needle on the rate of left limb regeneration compared to the rate of regeneration of noninfused right limbs. Progress of limb bud morphogenesis was determined daily. At each morphological stage there is a significant difference ($P < 0.05$, Student "t" test for related measures) in the number of days required by anti-NCAM-infused limb buds to complete that stage compared to contralateral, noninfused controls. There is no significant difference in the rate of regeneration between either normal rabbit Fab-infused or needle-inserted left limb buds and contralateral control limb buds. The consistent 3- to 4.5-day delayed growth of anti-NCAM-infused limb buds indicates that anti-NCAM postpones the onset of limb regeneration. Vertical lines denote SEM.

Table 1. Density of innervation in anti-NCAM-infused and control limbs

Fab infusion	Limb bud stage	PAD	Axons, no. 750 μm proximal to amputation level	Axons, no. 250 μm distal to amputation level
Normal	ME	14–16	1297 ± 103	238 ± 38
NCAM	ME	21	1277 ± 57	281 ± 99
Contralateral control	MB to Pal	21	1410 ± 29	586 ± 85

Data are expressed as mean ± SEM.

a loss of NCAM-mediated adhesion can be compensated for, in part, by some other type of adhesion. There are at least two mechanisms by which the antibodies could affect limb regeneration, the inhibition of nerve growth into the limb and/or the blocking of cell–cell interactions within the limb. Because NCAM mediates homophilic adhesion between cells, each of which express the molecule, the potential interactions include any combination of axons, mesenchymal cells, and epidermal cells.

The present results provide indirect evidence in support of a relationship between the neuronal influence on limb regeneration and the role of NCAM in cell–cell interactions. The delay in newt limb regeneration after exposure to anti-NCAM is similar to that seen after partial denervation (3, 4) and could be due to an inhibition of NCAM's ability to function as a preferred adhesive substrate for the growth of axons. Such a postponed axonal outgrowth could result from insufficient adhesion of growth cones to mesenchyme or the axon shafts of other neurons. Though the number of axons proximal to the amputation site in the experimental and control limbs was the same, the number of axons in the anti-NCAM-infused limb buds was substantially reduced compared to the contralateral controls. Thus, although there was no visualization of axon adhesion onto mesenchymal cells, it is likely that the anti-NCAM effect is manifested in and confined to the regenerating limb bud.

The possibility that slower axonal outgrowth results in delayed limb regeneration is strengthened by the observation that faster axonal outgrowth into the limb bud results in accelerated limb regeneration (5). The delay in limb regeneration, if due to inhibition of axonal growth, would support the suggestion that each stage of limb growth requires a minimum axonal quantity (5, 21, 25). The finding that the number of axons in anti-NCAM-infused limb buds at the ME stage was appropriate for this degree of regeneration rather than the length of time after amputation is also consistent with an effect of the antibody on the efficacy of axonal outgrowth.

Alternatively, it is possible that the delayed limb bud regrowth observed after treatment with anti-NCAM is due to interference with cell–cell interactions not directly involved in axon growth into the limb. For example, adhesion among NCAM-positive nonneuronal cells occurs during formation of limb tissue—i.e., mesenchyme–mesenchyme and epithelium–mesenchyme contact (26)—and might be disturbed by introducing anti-NCAM into the limb bud. Though the presence of an epithelium at every stage of limb regeneration is required (2), there are reports that suggest that continuous epithelium–mesenchyme contact is not essential in limb regeneration (27). Indeed, epithelium inhibits the differentiation of developing limb bud mesenchyme into chondrocytes though not into myoblasts (28).

The mesenchyme-like cells of the regeneration blastema appear to arise through dedifferentiation of cells at or near the amputation site. Schwann cells have been proposed as a possible origin of the blastema cells (26, 29) and their mitosis is believed to depend on interaction with nerve (26). In this respect it is interesting to note that NCAM is present on immature but not mature Schwann cells (30). Another possible source for the blastemal cells is the dedifferentiation of skeletal muscle cells at the amputation surface. Again, it has been found that embryonic but not mature muscle expresses NCAM (31–34).

In summary, the slowed growth of anti-NCAM-treated limbs is consistent with the possibility that these antibodies prevent interaction between daughter axons and mesenchymal cells by indirectly inhibiting axon elongation and/or directly by reducing axon–mesenchyme adhesion. To distinguish between these two mechanisms, it will be necessary to make more detailed observations on the behavior of growth cones and to assess the effect of anti-NCAM on axon–mesenchyme interaction in a more simple *in vitro* system (26).

This study was supported by Grant HD 18369 from the National Institutes of Health to U.R. and by Spinal Cord Society Grant CWIM-85 to I.G.McQ. Funding to M.S. from the Monsanto Corp., St. Louis, MO, is gratefully acknowledged.

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