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Fibre type specific expression of Leu19-antigen and N-CAM in skeletal muscle in various stages after experimental denervation

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Abstract. Leu-19 antigen, which seems to be identical with neural cell adhesion molecule (N-CAM), plays a major role in the innervation of muscle cells, and in adult muscle appears after denervation and during regeneration of muscle fibres, where it acts as part of a signalling system increasing the probability of re-innervation. This combined enzyme-histochemical and immunohistochemical study examined whether this signalling process was regulated in a uniform or differential pattern for type 1 and type 2 muscle fibres. The subscapular nerve of 18 rabbits was transsected with subsequent complete denervation of the supraspinatus muscle. Leu-19 and N-CAM immunohistochemistry was performed 2 to 64 days after surgery. Whereas in normal muscle there are virtually no Leu-19/N-CAM positive muscle fibres; from day 2 after denervation an increasing proportion of fibres expressed Leu-19/N-CAM, prior to any neurogenic atrophy. In the early stage of denervation Leu19/N-CAM expression was confined to type 1 fibres. After 11 days nearly all fibres were Leu19/N-CAM positive irrespective of their fibre type. Sixty-four days after denervation type 1 fibres became Leu19/N-CAM negative, while atrophic type 2 fibres showed intensive staining. Thus, expression of Leu-19 antigenity is differently regulated in both fibre types.

Key words: Neural cell adhesion molecule – Leu19 – Denervation – Rabbit

Introduction

The Leu-19 antigen was initially described as a surface glycoprotein on natural killer cells exhibiting non-major histocompatibility complex-restricted cytotoxicity. Monoclonal Leu-19 antibody cross-reacts with the surface of tumour cells and was identified on neuroendocrine and parenchymatous tissues, including nerve tissue and cardial myocytes (Griffin et al. 1983; Hercend et al. 1985; Lanier et al. 1986). Later on Leu-19 antigenity was also detected in skeletal muscle tissue: after labelling with a monoclonal antibody the surface of cultured myoblasts (Hohlfeld 1989) and satellite cells was Leu-19 positive in both normal and diseased muscle (Schubert et al. 1989). While positive muscle fibres are seen in degenerative and inflammatory myopathies, they are absent in normal muscle. Thus, the expression of the Leu-19 antigen in muscle-specific structures related to development and/or regeneration of muscle fibres has led to the conclusion that the Leu-19 antigen represents a molecular marker of muscle fibre regeneration (Schubert et al. 1989).

In recent years it has been shown that Leu-19 antigen is similar or identical with neural cell adhesion molecule (N-CAM; Lanier et al. 1988). Sequential immunoprecipitation and peptide mapping of N-CAM and Leu-19 on leukocyte and neuroblastoma cell lines revealed similar structures. N-CAM comprises a group of cell surface proteins playing an important role in the process of innervation of embryonic muscle (Rutishauer et al. 1983; Covault and Sanes 1985; Moore and Walsh 1985). In adult muscle it accumulates in the extra-cellular space and in muscle cells after denervation, and is supposed to increase the ease with which muscle is re-innervated (Covault et al. 1986).

Little is known about the fibre type specific pattern of Leu19/N-CAM expression in adult muscle. To investigate the time course of the expression of Leu19 and N-CAM in both fibre types after complete and irreversible denervation a combined enzyme-histochemical immunohistochemical examination was performed. ATPase-, NADH tetrazolium reductase (NADH-TR) reaction and Leu-19/N-CAM expression were examined in serial sections of denervated rabbit skeletal muscle from the second to the 64th day after resection of the nerve.

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Table 1.

Group	Time to sacrifice	Conventional histochemistry	Leu-19 immunoreactivity
A	2 days	Normal	Single fibre staining all type 2 fibres negative
B	5 days	Normal	About 30% fibres type 2 fibres negative
C	11 days	Slight atrophy of type 2 fibres	80–100% positive
D	21 days	Small angulated fibres	All fibres positive
E	34 days	Atrophic and few hypertrophic type 1 fibres	95% positive
F	64 days	Small group type 2 fibre atrophy and hypertrophic type 1 fibres	About 25–50% negative type 1 fibres

Materials and methods

After premedication with the tranquilizer phenothiazine (Combelen, Bayer, Germany) (1 ml per 3 kg weight s.c.) 18 male white New Zealand rabbits (over one year of age, life weight 4.5–5.0 kg) were anaesthetised with pentobarbital (Nembutal, WDT, Germany) (36–60 mg i.v.). Under aseptic conditions the suprascapular nerve was resected at the suprascapular sulcus to achieve complete denervation of the supraspinatus muscle. The minimal distance between both stumps of the nerve was 1 cm. Three animals were sacrificed at 2, 5, 11, 22, 34 and 64 days (groups A–F) after resection of the nerve (for details see table 1). Animals were killed by an overdose of barbiturate. An identical portion of the denervated supraspinatus muscle was removed from all animals and muscle specimens taken from the contralateral side served as controls.

Tissue samples were snap frozen in liquid nitrogen immediately after biopsy and cryosections of 10 µm thickness were stained with standard haematoxylin-eosin staining, myofibrillar ATPase pH 9.4, NADH-TR, basic and acidic phosphatase procedure (Dubowitz 1985).

For light microscopic demonstration of the Leu-19 and N-CAM antigen the indirect peroxidase technique was applied. Briefly, cross-sections were fixed in acetone for 10 min, air dried, rehydrated in TRIS-buffered-saline (TBS) and pre-incubated with 10% fetal calf serum in TBS for 15 min at room temperature to reduce non-specific background staining, followed by incubation with the primary monoclonal antibody for 1 h at room temperature. The Leu-19 monoclonal antibody (Becton Dickinson, Heidelberg, Germany) was used in a dilution of 1:30 and the N-CAM monoclonal antibody (Dakopatts, Hamburg, Germany) in a dilution of 1:50 as primary antibody. Both monoclonal antibodies can only be used for frozen tissue. After a 10 min rinse in TBS the secondary antibody (peroxidase-conjugated rabbit-anti-mouse; Dakopatts, Hamburg, Germany) diluted 1:50 was added for another 1 h incubation. Sections were washed again and incubated for 5 min with the substrate (containing 1 mg diaminobenzidine + 1 µl hydrogen peroxide/ml TBS). After the final rinse, the tissue was counterstained with haematoxylin, dehydrated and mounted in Eukitt (O. Kindler, Freiburg, Germany).

Conventional electromyographic examination was performed in all animals before sacrifice using a Neuromatic system (DANTEC, Danmark). For regristration of the signal concentric needle electrodes were used (Nicolet Instruments, USA).

Results

In normal supraspinatus muscle both Leu-19 and N-CAM immunoreactivity was restricted to a few tiny structures close to the outer side of the plasmalemma. These lacked nuclei, and thus might rather be nerve endings than activated satellite cells as judged on the light microscopic level. Occasional (i.e. less than 1%) muscle fibres stained positive with Leu-19 and N-CAM. Cross and longitudinal sections of nearly all muscle fibres were devoid of Leu-19/N-CAM immunoreactivity within or along the fibre. Muscle spindles, which were found in 5 biopsies, did not stain positive.

Muscle fibres were found to be polygonal or rounded on cross-section with few subsarcolemmal nuclei, which were not activated. Myofibrillar ATPase at pH 9.4 and NADH-TR reaction showed a slight predominance of type 2 fibres (bright staining in ATPase reaction and dark in NADH-TR reaction) with a normal mosaic of fibre type distribution. The mean diameter of type 2 fibres was slightly smaller than that of type 1 fibres. There were no specific abnormalities of the pattern of the sarcotubular system or the arrangement of mitochondria. With the basic and acidic phosphatase reaction no diagnostic enzyme activity was detectable within the muscle fibres.

Neurophysiological examination did not show signs of denervation.

By day 2 experimentally denervated supraspinatus muscle showed that about 5%–10% of the muscle fibres became Leu-19 positive. By day 5, this value had reached 25–40%. On serial sections the localization and the pattern of expression of Leu-19 reactivity was identical with N-CAM expression.

At this stage Leu-19/N-CAM positive fibres were arranged over the cross-section in a mosaic pattern ressembling single motor units, that comprised an estimated 10 fibres. Immunoreactivity was most prominent over the whole length of the sarcolemma, with some patchy accumulation, probably close to the neuromuscular junctions. Somewhat weaker Leu-19/N-CAM immunoreactivity was distributed in a reticular pattern throughout the fibre, presumably at the inter-myofibrillar network. Leu-19/N-CAM positive myonuclei or resting satellite cells could not be identified. Intra-fusal fibres of the muscle spindles were Leu-19/N-CAM positive with the corresponding nerve twigs, as were all intra-muscular nerves. The connective tissue and blood vessels were devoid of Leu-19/N-CAM immunoreactivity.

With conventional histological techniques including enzyme-histochemistry (ATPase, NADH-TR, acid and alkaline phosphatase) the Leu-19/N-CAM positive fibres looked completely normal at this stage of denervation (day 2 and 5). The myonuclei were of normal number and structure without any signs of activation; central displacement of nuclei was not seen.

All fibres that were Leu-19/N-CAM-positive by day 5 showed low enzyme-histochemical activity of myofi-

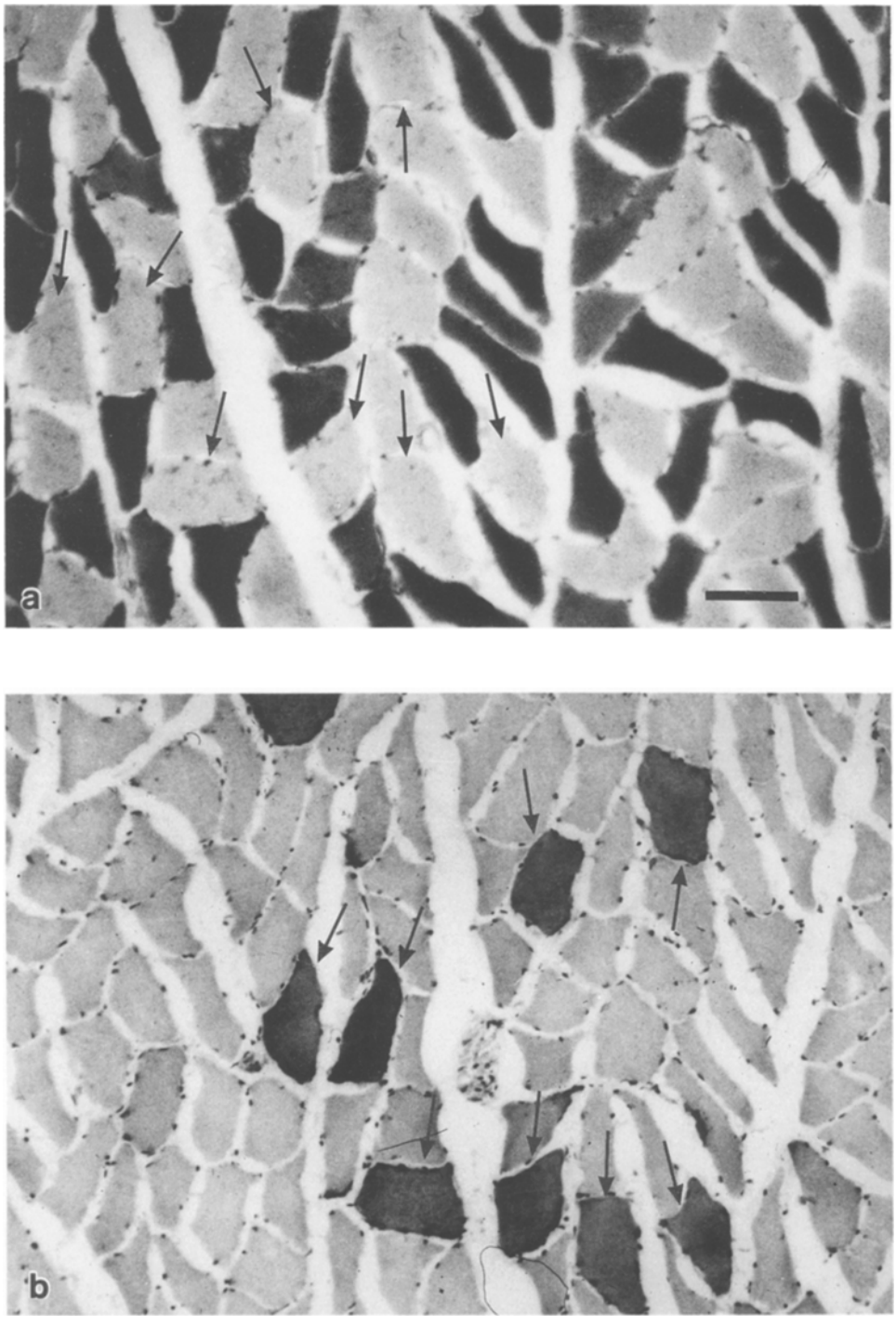

Fig. 1a, b. Selective expression of Leu-19/N-CAM in type 1 fibres 5 days after denervation (**a**, myofibrillar ATPase reaction; **b**, Leu-19; bar = 50 μm)

brillar ATPase and high activity of NADH-TR, that is, slow twitch type 1-fibres first expressed the Leu-19/N-CAM antigen (Fig. 1a, b). During the first days after denervation neither a neurogenic atrophy nor small angulated fibres staining dark with NADH-TR or target fibres could be detected.

With time from denervation the proportion of Leu-19/N-CAM positive fibres increased further: after 11 days about 80%, after 3 weeks and 34 days nearly all fibres were positive except single ones, that were Leu-19/N-CAM negative. These fibres showed regressive changes, mostly necrosis, with an increase of acid phosphatase activity. Cellular infiltrates adjacent to necrotic muscle fibres did not express the Leu-19/N-CAM antigen, which is a marker for natural killer cells, too.

From the 11th to the 22nd day after denervation, immunoreactivity was distributed within positive fibres similar to the pattern that was visible during the first days after denervation. However, the intensity was not completely uniform throughout the cross-section: from day 11 on an increasing number of slightly to moderately atrophic fibres distributed in a random manner were

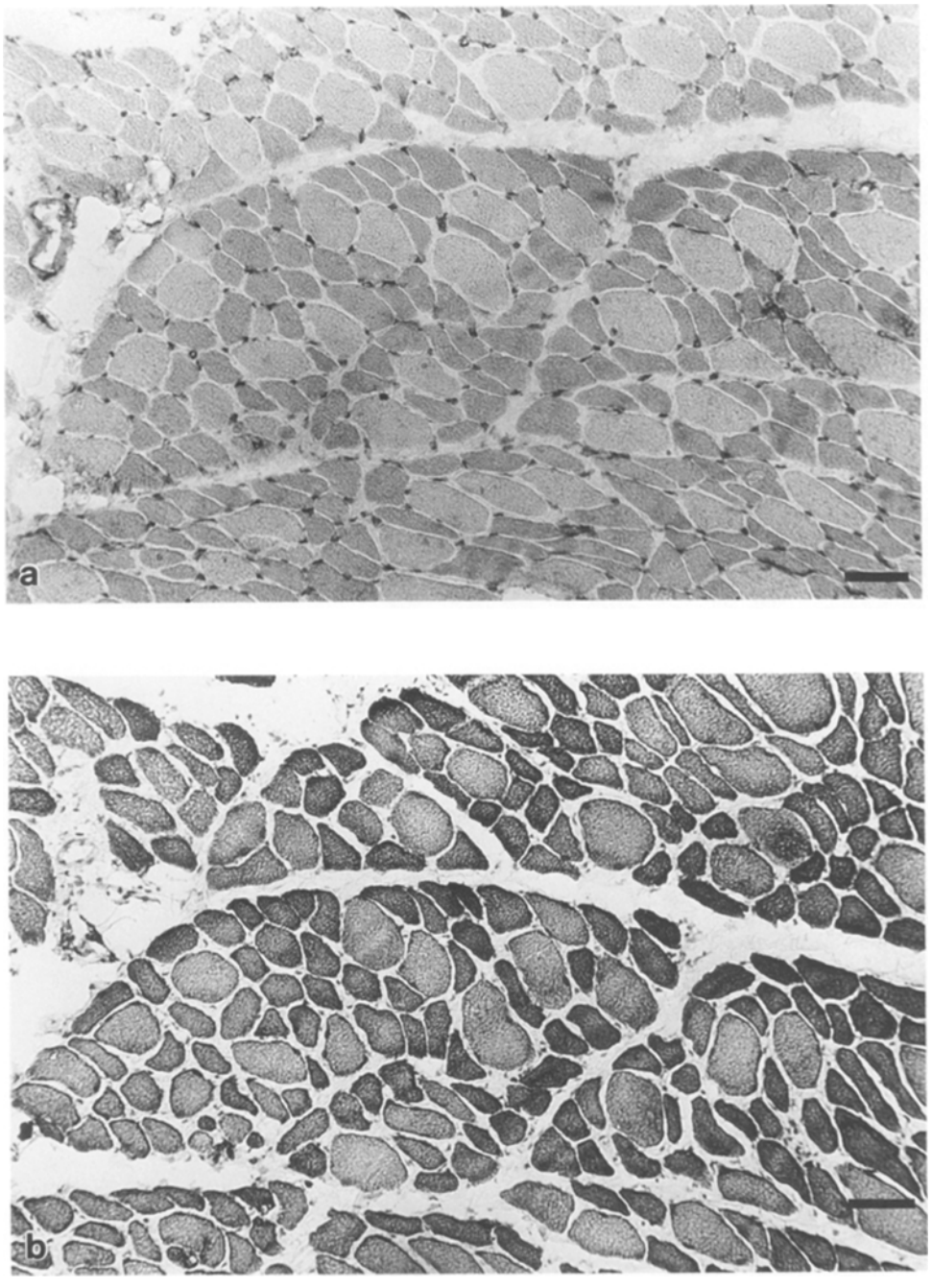

Fig. 2a, b. Low expression of Leu-19 in slightly hypertrophic type 1 fibres 34 days after denervation (**a**, myofibrillar ATPase reaction; **b**, Leu-19; bar = 50 μm)

more intensely stained, especially close to the sarcolemma, than other fibres of about normal size. These were more homogeneously and less intensively marked.

During the first three weeks after denervation conventional techniques did not disclose a typical grouped neurogenic atrophy. With the NADH-TR reaction a few small angulated fibres showed up after 21 days. These were intensely stained with the Leu-19/N-CAM antibody giving the impression of a condensation of sarcolemmal and inter-myofibrillar immunoreactivity. Target fibres were not seen.

After 34 days the ATPase and NADH-TR enzyme reactions showed moderately atrophic, flattened and elongated type 2 fibres and rounded normal sized or moderately hypertrophic type 1 fibres distributed in a random fashion. At this stage it became obvious that the strongly Leu-19/N-CAM reacting fibres were of the enzyme histochemical type 2. Most of the fibres with low expression of Leu-19/N-CAM were normal sized or moderately hypertrophic type 1 fibres (Fig. 2a, b).

With the myofibrillar ATPase reaction no type grouping was present. An activation of the subsarcolemmal nuclei could be seen in atrophic fibres, but no terminal atrophic fibres were present. The denervated muscle fibres did not disclose any structural changes. The haematoxylin-eosin stain and the alkaline and acid phosphatase enzyme reaction revealed no signs of re- or degeneration.

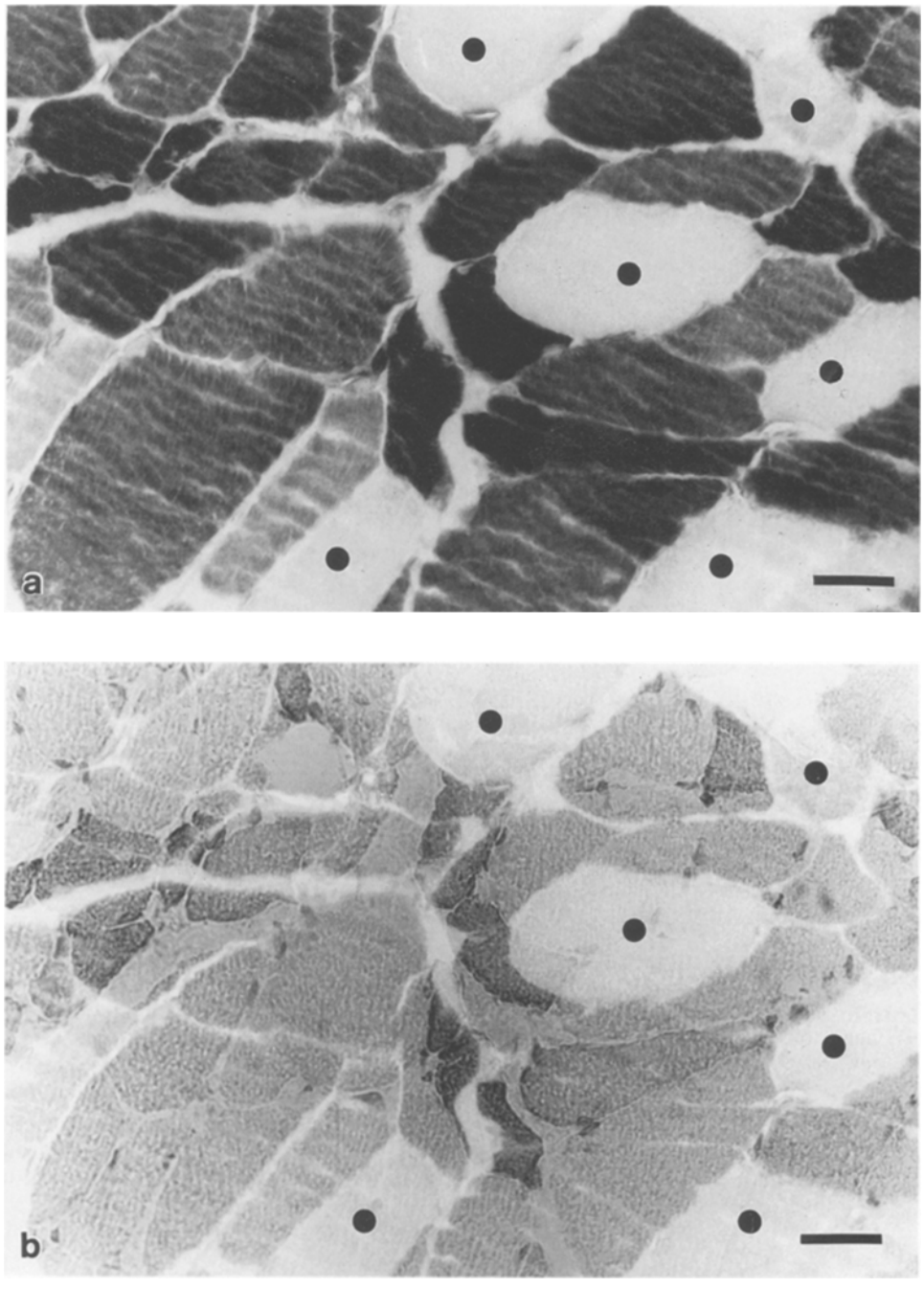

Fig. 3a, b. Absent Leu-19 staining in type 1 fibres, intense expression in atrophic type 2 fibres 64 days after denervation (**a**, myofibrillar ATPase reaction; **b**, Leu-19; bar = 20 μm)

In muscle specimens taken 64 days after denervation the changes were more pronounced when compared with that at 34 days. The ATPase reaction revealed islets of hypertrophied rounded type 1 fibres surrounded by a network of atrophic type 2 fibres arranged in small groups. There was no type grouping phenomenon.

The hypertrophic type 1 fibres were now completely Leu-19/N-CAM negative. These fibres were surrounded by Leu-19/N-CAM positive type 2 muscle fibres. Staining of normal sized type 2 fibres was less intense than that of atrophic ones (Fig. 3a, b).

Neurophysiological examination revealed signs of denervation as early as day 11. There was no evidence of reinnervation.

Discussion

Our experiments prove that the Leu-19 and N-CAM antigen is expressed on muscle fibres as early as day 2 after experimental denervation and reaching its maximal expression after 11 days. On serial sections Leu-19 and N-CAM stained identical muscle fibres. This supports the view that there is either a cross-reaction of antibodies against N-CAM and Leu-19-antigen or that both glycoproteins are similar or identical. This finding is in accordance to the results of Lanier et al. (1988), who found a similarity or identity of both molecules in neuroblastoma cell lines and leucocytes.

N-CAM staining appeared in a diffuse distribution

in the cytoplasma and exact localization within the cell is not possible by light microscopy. Electron microscopic studies revealed that at the time when myotubes separated and matured into myofibres, N-CAM could be detected in intra-cellular structures ressembling T-tubes (Covault and Sanes 1986). No data are available about ultrastructural changes of N-CAM distribution after denervation. A similar spatio-temporal distribution to that in our study has been reported in rat diaphragm using an antibody against N-CAM (Covault and Sanes 1985) with first expression of N-CAM after 2 days and a maximum 4–8 days later.

Conventional histochemistry did not disclose a neurogenic atrophy until day 34, and the pattern typical for a lesion in the proximal part of the motor neuron was not seen until day 64 when small groups of slightly hypertrophied type 1 fibres surrounded by a network of atrophic type 2 fibres became apparent. The findings of this study are in accordance with the results of Schubert et al. (1989) and Covault and Sanes (1985), indicating that Leu-19/N-CAM expression is generally not restricted to a certain fibre type. Between day 11 and 22 after denervation a highly increased expression of Leu-19/N-CAM could be observed in both fibre types.

Nevertheless, at very early and late stages during the process of denervation there is a selective expression of Leu-19/N-CAM. Up to day 5 expression was confined to type 1 fibres, while a positive reaction of nearly all fibres was found after 3 weeks, when type 1 fibres stained less well than type 2 fibres. Sixty-four days after denervation type 1 hypertrophy was seen with a loss of Leu-19/N-CAM expression, however, there was a further increase of Leu-19/N-CAM expression in atrophic type 2 fibres. Hypertrophy of type 1-fibres that have become independent of neural control after denervation has been described by others (Sola et al. 1973) and experimental data suggest that the traction of the muscle is responsible for this effect. No data are available on the regulation of expression of Leu-19/N-CAM by mechanical factors or by the trophic status of muscle cells.

Our findings indicate that there is a fibre type specific regulation of Leu-19/N-CAM expression at least at some stages during denervation. While Leu-19/N-CAM can be found earlier in type 1 than in type 2 fibres, it persists for a longer time in type 2 than type 1 muscle fibres. Studies published have revealed a number of different mechanical and biochemical reactions after denervation for both fibre types. After transection of the sciatic nerve in guinea pigs type 2 fibres atrophied twice as fast as type 1 fibres during the first 17 weeks (Kraft 1990). There is a slow to fast shift of mechanical properties following denervation (Ansved and Larsson 1990). The enzymes phosphoglycerate mutase and creatine phosphokinase decreased more markedly in fast twitch than in slow twitch muscles (Andres et al. 1990). An over-production of acetylcholinesterase was found during reinnervation of the rat soleus muscle, which is composed of slow-twitch type 1 fibres, but not in the extensor digitorum brevis, which is a fast-twitch muscle (Misulis and Dettbarn 1985). Though the exact mode of action of N-CAM during denervation is not known, the data presented suggest that N-CAM may be part of the signaling system leading to some of the differences between both fibre types.

Irrespective of the fibre type affected there was a decline of Leu-19/N-CAM expression at later stages after denervation. Since the distance between both nerve stumps was more than 1 cm in all our animals and no neurophysiological signs of re-innervation could be detected, this decline cannot be explained by re-innervation. This suggests that denervation is not the only factor influencing Leu-19/N-CAM expression.

Two different functions of the molecule have been proposed. Leu-19 has been identified on satellite cells that are the stem cell pool of muscle regeneration, on proliferating myoblasts, myotubes both in vivo and in vitro (Hohlfeld 1989; Schubert et al. 1989) and on tumour and natural killer cells (Nawrotzki et al. 1990). Therefore Leu-19 was first associated with cell proliferation and muscle fibre regeneration (Schubert et al. 1989). It might be a functional component of a membrane recognition system required for myoblast fusion in addition to correcting longitudinal attachment of myotubes to intact fibre ends. However, Nawrotzki and co-workers screened neuroectodermal malignancies and saw Leu-19 on all tumour cells, whereas Ki-67, a molecule specific for cell proliferation, was restricted to a small proportion of the tumour cells (Nawrotzki et al. 1990) which suggests that the expression of Leu-19 is independent of cell proliferation and these findings are in accordance with our results. There was no histological evidence of cell proliferation or regeneration, such as basophilia or activated nuclei, proliferating satellite cells, or an increase of alkaline phosphatase in experimentally denervated Leu-19 positive rabbit muscle fibres. We could not identify Leu-19 positive proliferating satellite cells in the control or denervated supraspinatus muscle by light microscopy. The patchy accumulation at the edges of the membrane, without the presence of a nucleus, probably marks the neuromuscular junction. In addition to positive muscle cells we also saw intramuscular unmyelinated nerve twigs, in both normal and deseased muscle, that are Leu-19 positive (Schubert et al. 1989) and do not proliferate.

A second important function has been proposed for the Leu19/N-CAM molecule. It seems to play, among other functions, a role in the formation of plexiform layers, neurite fasciculation, and nerve-muscle interaction (Rutishauser 1984; Edelmann 1986). N-CAM seems to be needed for proper nerve-muscle connections (Rutishauser et al. 1983). It is not seen in normal muscle fibres, but is expressed at the motor end plate after denervation (Walsh et al. 1987) and within regenerating muscle fibres (Moore and Walsh 1985). N-CAM disappears with experimental re-innervation (Covault and Sanes 1985). Muscle biopsies from patients with neurogenic atrophies showed N-CAM labelled fibres in neurogenic atrophies (Cashman et al. 1987). The absence of Leu-19/N-CAM in necrotic fibres indicates that the expression of Leu-19 is an active process that requires an intact muscle fibre.

Thus, Leu-19/N-CAM may be of practical value in

experimental settings as well as the diagnosis of early stages of neurogenic muscular disorders that have not yet led to muscular atrophy. It might give insight into neural involvement in certain myopathies. The fact that at later stages following denervation Leu-19/N-CAM reactivity is mainly expressed in atrophic fibres may provide a tool for assessing the dynamics of denervation. Both the total number of Leu-19/N-CAM positive fibres, the fibre size and the fibre type have to be taken into account, in order to distinguish diseases with rapid denervation from those with a more chronic course. Further studies have to evaluate if a combined morphometric/immunohistochemical examination can help to define the prognosis of some neuromuscular diseases such as various forms of spinal muscular atrophies.

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