

Comparative Studies of Hind Limb and Diaphragm Muscles of *mdx* Mice

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Abstract

Histological, immunocytochemical and immunochemical characteristics of diaphragm muscle from *mdx* and control mice (14-days to 12-months of age) were compared to those of their hind limb muscles. In contrast to *mdx* hind limb muscles regeneration after necrosis, which was evident after one month of age, does not restore the diaphragm muscle structure. In contrast also to *mdx* hind limb muscles in the diaphragm there was a significant extracellular matrix proliferation, which was manifested starting from 6 months of age of the animals by increased intensity of fibronectin and collagen IV immunostaining. In immunocytochemical studies with anti-C terminus and anti-N terminal domain antibodies no staining of dystrophin was found in the *mdx* diaphragm, in hind limb *mdx* muscles rare positively stained fibres were observed starting from 3 months of age of the animals. The Western blotting technique in normal mice diaphragm and hind limb muscles revealed dystrophin bands of equal intensity with anti-mid rod and anti-C terminus antibodies. In *mdx* samples the staining with anti-C terminus antibodies was evidently weaker. The anti-mid rod antibodies detected dystrophin in the *mdx* diaphragm between the 3^r and 6^t months of age (2 to 5 percent of normals), before and afterwards dystrophin was absent or appeared in traces. In hind limb *mdx* muscles traces of dystrophin were present up to the 3^r month of age of the animals, then after the content of dystrophin was between 8 and 20 percent of normals (anti-mid rod antibodies).

The studies indicate that dystrophin deficiency in hind limb muscles and diaphragm of *mdx* mice is present already before the degeneration/regeneration processes are appearing. The presence of dystrophin positive fibres in the hind limb muscles of adult animals are likely to represent revertant fibers and clones of somatic reversion. The *mdx* diaphragm is more similar as compared to *mdx* skeletal muscles to Duchenne's dystrophy skeletal muscles. Whatever the role of dystrophin it is not crucial for muscle recovery and solely it is not determining the clinical and morphological manifestation of dystrophy.

Key words: *mdx* mice, hind limb muscles, diaphragm muscle, dystrophin, fibronectin, collagen IV.

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The *mdx* mouse represent an X-linked recessive myopathy characterized by raised plasma enzymes activities [3], lack of dystrophin in hind limb muscles [1, 8, 9, 25], and a point mutation in the dystrophin gene [21]. Low expression of dystrophin of an approximately normal size in skeletal muscles of mutants *mdx* 3Cv mice is described [5]. Despite the lack of dystrophin the *mdx* mice either do not show obvious functional disability [3, 22], or they express only mild muscle weakness [16, 24], sometimes appearing in the form of muscular tremor and mild movement incoordination in old animals [2]. There were no examinations of dystrophin in *mdx* diaphragm performed, yet. Although no overt respiratory impairment is present

there are some diaphragm functional changes including reductions in strength, elasticity, twitch speed and the fibre length [8, 22]. There are striking differences in morphological appearance between hind limb and diaphragm muscles in *mdx* mice, degeneration/regeneration being present in the former, in the latter this process is accompanied by connective tissue and adipose tissue replacement [2, 6, 11, 14, 19, 21, 22].

It was of value to document the content of dystrophin in *mdx* diaphragm before the disease process becomes evident and follow it up in the course of life of the animals in comparison to their hind limb muscles. This could indicate

Comparative studies of hind limb and diaphragm muscles of mdx mice

on the role of dystrophin for muscle recovery and the appearance of dystrophic changes.

Materials and Methods

Male mutant C57BL/10 *mdx* mice, 14 days to 12 months of age, and control age-matched normal mice of C57BL/6J strain, were used in the studies. Animals (three or four in each group) were fed a standard laboratory diet, they were killed by cervical dislocation in ether anaesthesia and extensor digitorum longus (EDL), gastrocnemius and diaphragm muscles were carefully and rapidly removed. Cryostat sections (8 μ m) were stained according to standard techniques. For immunohistochemical studies dystrophin anti-N terminal and anti-C terminus domain antibodies, diluted 1:250, (a generous gift of Dr. E.P. Hoffman, University of Pittsburgh) were used. To check the possible involvement of muscle basal lamina and the extracellular matrix into the process of fibrosis the following antibodies were applied: anti-fibronectin (1:100, Daco) and anti-collagen IV (1:60, Daco). The immunostaining of tissue sections were performed on fresh frozen muscle sections, using indirect immunofluorescence methods.

For Western blotting examinations the gastrocnemius and diaphragm muscles were frozen quickly in dry ice and preserved in this temperature until used. After thawing all procedures were conducted at +4°C. The samples were weighed, finely minced with scissors and homogenized in 20 volumes of buffer containing 75 mM Tris-HCl (pH 6.8), 15% (w/v) SDS, 20% (v/v) glycerol, 5% (v/v) β -mercaptoethanol and 0.001% (w/v) bromophenol blue [17]. The concentration of SDS-solubilized proteins was estimated by the method of Peterson [20]. The samples were heated in a boiling water for 3 min, a volume corresponding to 30 μ g of protein were analysed on 1.0 mm thick SDS-polyacrylamide gels using the system of Laemmli [13]. Mini-Protein II ready 4-15% gradient gels (Bio Rad) were used. The separated proteins were blotted on nitrocellulose membranes 0.2 μ m using 0.25 M Tris-HCl, 1.92 M glycine, 20% methanol buffer (pH 8.3) overnight at 30 V, 40 mA (Mini-Protein II Electrophoresis Cell and Mini Trans-Blot Electrophoretic Transfer Cell, Bio Rad). Unreacted binding sites on dry nitrocellulose sheets were blocked with 5% non-fat dry milk in 10 mM Tris-HCl, 0.15 M NaCl and 0.05% (v/v) Tween 20 (pH 7.5), which was also used to wash the nitrocellulose sheets after blocking and incubation with antibodies. Incubation with primary antibodies lasted 3 hours. Monoclonal anti-mid rod and anti-C terminus dystrophin antibodies (1:300, Novocastra Lab.) and secondary antibodies (alkaline phosphatase conjugate goat anti-rabbit IgG, streptavidin-biotinylated, 1:300, Bio Rad) were applied. The procedure according to Amplified Alkaline Phosphatase Immuno-Blot Assay Kit (Bio Rad) was followed. Wet nitrocellulose sheets were processed further by EC910 densitometer (Fisher) and EC934 version 2.0 software 486 computed.

20 μ g of proteins were also run in the same conditions as above and stained with Coomassie Brilliant Blue R 250 in order to separate muscle contractile proteins. This may serve as an additional approximate indicator of the amount of loaded proteins.

Results

In the 14 days old *mdx* mice, both extensor digitorum longus (EDL) and diaphragm muscles appeared to be normal. At 1 month of age myofibre degeneration, necrosis and regeneration in EDL were observed. Necrosis with phagocytic cells infiltration affected large clusters of EDL fibres (Fig. 1a). Regenerating fibres presented as entirely small myotubes formed large fascicles. At 3 months of age in EDL the degenerative process was more marked, the variability of muscle fibre size was greater. At the age of 6 months more advanced changes were present. About 80% of muscle fibres had centrally located nuclei, there was a number of hypertrophic fibres present, fibre splitting was also observed (Fig. 1b). At the age of 12 months in EDL muscles the percentage of fibres with central nuclei was higher than before and almost all muscle fibres had centrally located nuclei (Fig. 1c). Hypertrophy and fibre splitting were more pronounced. Necrotic fibres were still present.

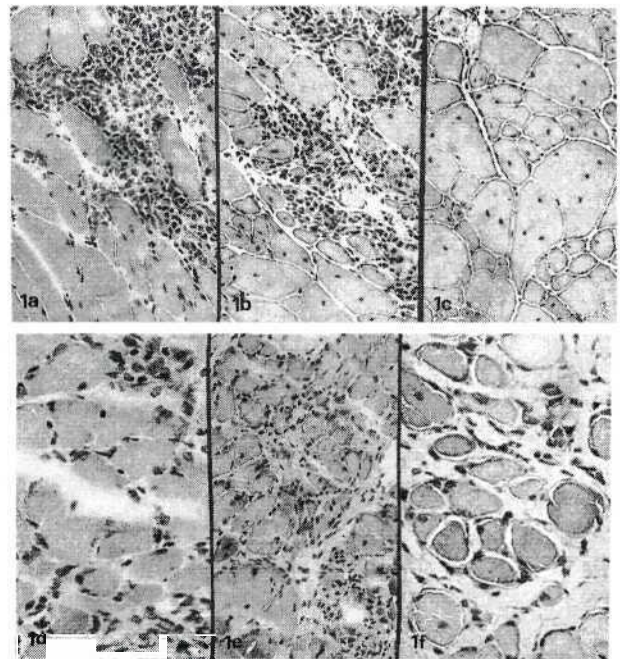


Figure 1. Histological examination of EDL muscle (a-c) and diaphragm (d-f) of *mdx* mouse at the age of 1, 6, and 12 months. HE x 448. In EDL there are numerous necrotic fibres undergoing phagocytosis (a-b). Large hypertrophied muscle fibres are mixed with small fibres. Note the central position of nuclei (c). In diaphragm small area of necrotic fibres (d) at the age of one month is present. An increased amount of endomysial connective tissue and loss of muscle fibres at age 6 and 12 month (e, f) is evident.

Comparative studies of hind limb and diaphragm muscles of *mdx* mice

In contrast to EDL the necrotic fibres in diaphragm did not occur in large groups, they died in small fascicles, and regenerating fibres were scarce. Diaphragm indicated at the same time progressive degeneration, wide variation of fibre size and structure changes. Evidence of regenerative activity persisted as fibres with central nuclei. A progressive extensive loss of the fibres and replacement by connective tissue was observed. The remaining fibres differed in size and structure, they were ensheated in a dense extracellular matrix (Fig. 1d-f).

In young *mdx* animals dystrophin was not detected in both investigated muscles (Fig. 2). In EDL dystrophin positive fibres, single or clustered in groups, were found occasionally. The number of dystrophin positive fibres in EDL progressively increased with age (Fig. 2a-d). In diaphragm the total absence of dystrophin persisted up to 12 months of age (Fig. 2e-h).

In *mdx* EDL fibronectin (Fig. 3a-c) and collagen IV staining (Fig. 4a) was normal up to 12 months of age. In *mdx* diaphragm a significant extracellular matrix proliferation manifested by an increase of fibronectin (Fig. 3e-f) and collagen IV (Fig. 4b) was present.

When dystrophin was tested by the Western blotting technique with the anti-mid rod dystrophin domain antibodies only traces were observed in *mdx* hind limb muscles up to 3 months old animals, from 3 to 12 months of age mice the dystrophin content was between 8 and 20 percent of normals (Fig. 5). Anti-C terminus dystrophin antibodies did not detect dystrophin in hind limb muscles up to 3 months of age, afterwards up to 12 months of age dystrophin appeared in traces. In *mdx* diaphragm the anti-mid rod domain antibodies either did not detect dystrophin, or this protein appeared in traces in all animals except of the 3 and 6 months old animals, where the dystrophin band was 2 to 5 percent of normals (Fig. 6). The anti-C terminus antibodies did not detect dystrophin in the diaphragm of all *mdx* animals. Both in control and *mdx* hind limb muscles and diaphragm the size of dystrophin was about 400 kDa. Using the mid rod domain antibodies "degradation" bands at 220 kDa were observed in all muscle preparations. No such bands were present when the C terminus antibodies were applied. Examination of muscle protein profiles, visualized by Coomassie Blue stain, revealed comparable

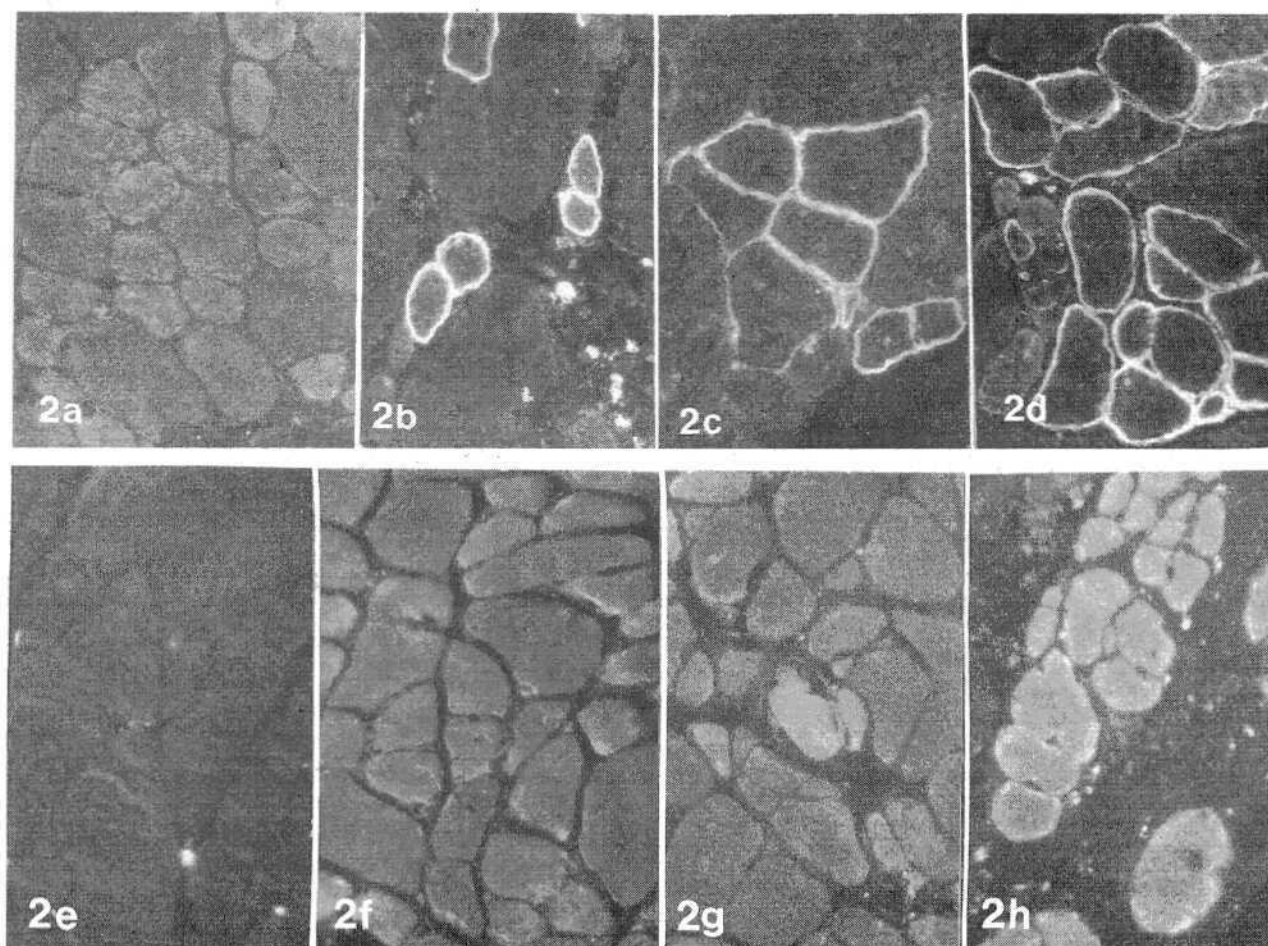


Figure 2. Immunohistochemical labelling of dystrophin in EDL muscle (a-d) and diaphragm (e-h) of *mdx* mice at the age of 1, 3, 6 and 12 months. $\times 448$. In EDL total absence of dystrophin labelling (a), occasional decorated fibres (b) and a progressive increasing number of dystrophin-labelled muscle fibres (c, d) is present. The diaphragm shows a total absence of dystrophin labelling (e, f, g, h).

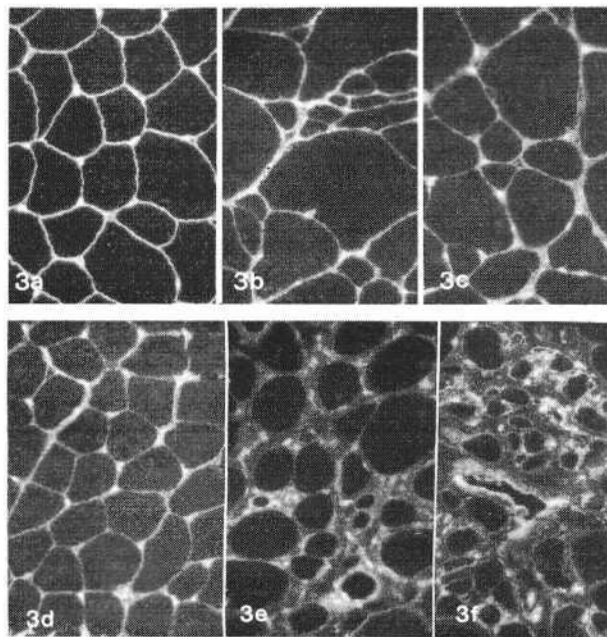


Figure 3. Immunohistochemical labelling of fibronectin at the age of 1, 6, 12 months. EDL muscle (a-c) and diaphragm (e-f) of mdx mouse. Normally labelled EDL muscle fibres (a-c) increasing amount EDL of fibronectin in diaphragm (e-f) with the age of the animals. $\times 448$.

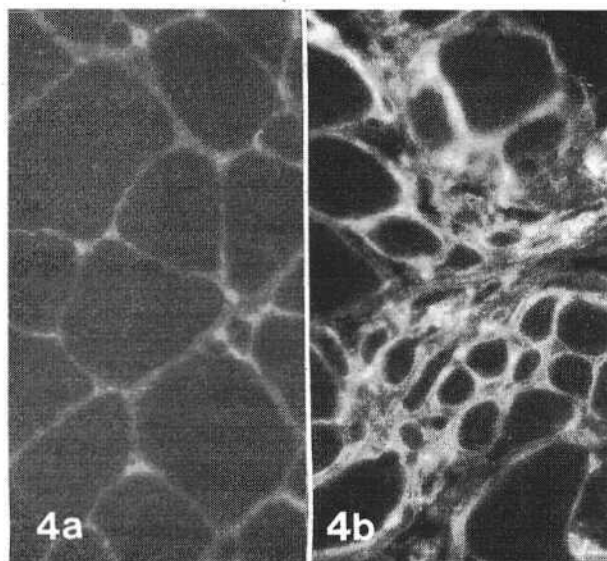


Figure 4. Mdx mice. Immunolabelling of collagen IV in EDL muscle (a) and diaphragm (b) of mdx mouse at the age of 12 months. $\times 448$. Note the increased amount of collagen in the diaphragm.

intensity of protein bands in all tested muscle (Fig. 5, Fig. 6).

Discussion

Several morphological and biochemical reports on mdx hind limb muscles are already published [1-4, 6, 7, 9-11,

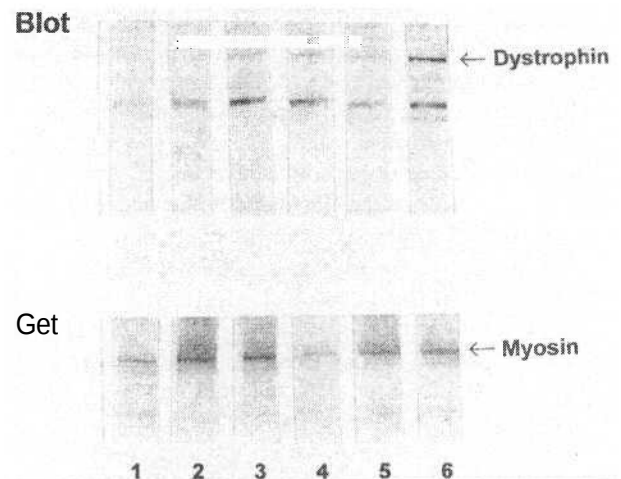


Figure 5. Western blotting of dystrophin of hind limb muscles in mdx and normal mice. 1-14 days, 2-30 days, 3-60 days, 4-90 days, 5-120 days old mdx mice, 6-30 days old normal mice. Note traces of dystrophin up to three months of age of the mdx animals, afterwards the dystrophin band is more abundant. In all preparations a „breakdown” product at 220 kDa is visible. Dys 1 (Novocasta Lab) mid rod antibodies were used. The lower panel of lanes represent PAGE electrophoresis of muscle contractive proteins as an approximate indicator of the amount of loaded proteins.

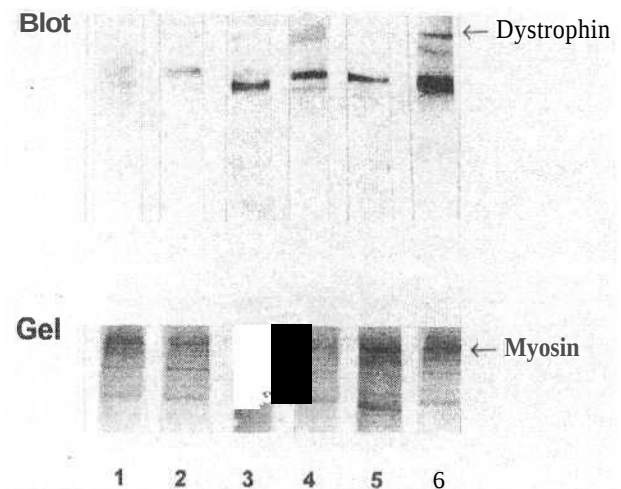


Figure 6. Western blotting of dystrophin of diaphragm in mdx and normal mice. 1-14 days, 2-30 days, 3-60 days, 4-90 days, 5-120 days old mdx mice, 6-30 days old normal mice. Note the absence of dystrophin in all mdx animals except of those at the age between 3 and 6 months, where dystrophin traces are present. In all preparations a "breakdown" product at 220 kDa is visible. Dys 1 (Novocasta Lab) mid rod antibodies were used. The lower panel of lanes represent PAGE electrophoresis of muscle contractive proteins as an approximate indicator of the amount of loaded proteins.

13, 14, 16, 19-27]. Only few studies of *mdx* mouse diaphragm are reported, yet [8, 14, 22]. In the hind limb muscles degeneration followed by regeneration with a cranio-caudal trend in developing muscle necrosis is reported [16]. Fibrosis is present only in very old animals [19]. A marked accumulation of collagen was also, however, found in endomysium and perimysium in skeletal muscles of young *mdx* animals [15]. Muscle necrosis may occur after 2 to 3 weeks of age, afterwards regeneration occurs and restores the muscle structure. In contrast to *mdx* mice hind limb muscles regeneration after necrosis fails to restore the diaphragm muscle structure and its function and fibrosis occurs very early [6, 8, 14, 19, 22]. According to some authors fibrosis in the *mdx* mice diaphragm is not present up to the 3 months of age [6, 22].

Our observation on the degenerative/regenerative process in hind limb muscles of *mdx* mice are generally similar to that reported in the already mentioned studies. We did not observe necrosis to be present at the age of 14 days, at 30 days of age necrosis was followed by successful regeneration. The *mdx* EDL muscles show a continuous ability to spontaneous regeneration with the presence of the large areas of newly formed fibres. On the contrary the *mdx* diaphragm is loosing its regenerating capacity, extensive fibrosis occurs as early as 6 months of age. The extensive hyperplasia of connective tissue, as well as the gradual failure of regenerative process, differs the diaphragm from EDL muscle in the *mdx* mice.

The mechanism(s) which are activating the regenerative response in diseased muscle fibres are not known, yet. One of the possibilities is that overproduction of basal lamina components, such as fibronectin and collagen IV observed in the diaphragm of *mdx* mice, may alter extracellular matrix structure and inhibit the muscle fibre regeneration. This is not the case in the *mdx* EDL muscles, and so the regenerative process may be active. However, the explanation of the regenerative process inhibition by extensive proliferation of fibrous tissue should be taken into account with reservations.

Another aspect of our study was the finding of scattered individual fibres or clusters of fibres in the *mdx* EDL muscle expressing dystrophin. The number of dystrophin positive fibres was increasing with age of the *mdx* animals. Dystrophin positive fibres were presented also before in hind limb muscles of the dystrophic mice [1, 7, 10], they responded to the immunocytochemical ones in muscles of Duchenne's dystrophy patients [12, 18, 19]. The presence of the dystrophin positive fibres represent a somatic reversion or suppression of the *mdx* mutation [10]. A second site mutation which prepares an in-frame deletion is also discussed. Grouping of the dystrophin positive fibres in EDL muscle may be attributable to extensive fibre splitting, as well as to mutation in satellite cells, supplying dystrophin-positive nuclei to a group of contiguous fibres.

This is the first report on dystrophin content in the *mdx* diaphragm both immunocytochemically, as well as immunochemically. In diaphragm dystrophin was absent in all

mdx animals when immunocytochemical methods with anti-C terminus and anti-N terminal domain dystrophin antibodies were used. No fibres or cluster of fibres dystrophin positive, as in the EDL muscles, were present. When the Western blotting technique and the anti-mid rod antibodies were applied to the diaphragm samples after 3 to 6 months of age of the animals dystrophin was present in traces. Lack of dystrophin staining with anti-C terminus antibodies, especially in the diaphragm muscle, may indicate that the dystrophin's C-terminus is more affected, or more easily degraded during the sample preparations than its rod domain.

Diaphragm in *mdx* mice is evidently more affected than the hind limb muscles, possibly because of its ventilatory overload. The *mdx* diaphragm resembles more the dystrophic process in human dystrophy than the *mdx* hind limb muscles. Whatever the role of dystrophin, this protein seems to be not necessary to start and continue the process of muscle structural and functional regeneration. Lack of this protein, seems also not to be the determinant factor of the appearance of dystrophic changes.

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Comparative studies of hind limb and diaphragm muscles of mdx mice

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