

Muscle Regeneration in *mdx* Mice: Resistance to Repeated Necrosis is Compatible with Myofiber Maturity

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Abstract

The regenerated muscle fibers of *mdx* dystrophic mice remain permanently centronucleated and show resistance to repeated necrosis. Searching for mechanisms that could induce such resistance, we studied whether *mdx* regenerated fibers differ from the original ones by having a smaller calibre or by showing a different histochemical typing. Moreover, since in several genetic diseases of skeletal muscle the deleterious effects of abnormal gene expression are suppressed in immature fibers, we studied the expression of developmental myosin heavy chain (d-MHC) as a marker of persistent immaturity in *mdx* regenerated fibers. Our results show that in *mdx* gastrocnemius: 1) the course of the disease is accompanied by a prevalent loss of large-calibre original fibers with relative preservation of small-calibre fibers. On the contrary, regenerated fibers tend to grow larger than the original ones, without showing repeated necrosis. 2) Type II fibers show a mild decrease in number but are still largely preponderant over type I fibers at 6 months. 3) Expression of d-MHC is absent in fully regenerated myofibers. In conclusion, while large calibre and type II fibers are more vulnerable among the original myofiber population, these factors do not interfere with viability of regenerated fibers. Therefore, in *mdx* regenerated fibers, the effects of abnormal gene expression are attenuated through undetermined mechanisms that may be related to centronucleation but do not imply a permanent myofiber immaturity.

Key words: *mdx* mouse; muscle regeneration; muscle morphometry; ATPase histochemistry; developmental myosin heavy chain.

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Duchenne muscular dystrophy (DMD) and *mdx* mouse dystrophy are both caused by a genetic defect of dystrophin, a structural protein associated with the sarcolemma [16, 35, 39], but they show a partially different phenotype.

Indeed, DMD muscle presents recurrent necrosis, defective regeneration with progressive loss of myofibers, and fibro-fatty substitution. In *mdx* mice, apart from diaphragm which shows DMD-like features [34], limb muscles present a transient period of widespread myofiber degeneration between 3 and 12 weeks of age, followed by full regeneration and recovery of function. Subsequently, only small groups of necrotic fibers can be seen, probably belonging to the residual population of original fibers. By 6 months of age, a large majority of fibers are regenerated and hypertrophic fibers are present, whereas adipocyte proliferation and fibrosis do not occur [1, 5, 8, 36]. However, late in life, *mdx* muscles show more evident dystrophic changes, with muscle atrophy and endomysial fibrosis [21, 33]. Thus, while DMD is a progressive disorder,

mdx dystrophy is a multi-staged disorder [27], with a spontaneous attenuation of muscle pathology enduring for most of the mice's life. This can explain why these animals present only a transitory motor impairment [29] and a near-normal lifespan [21].

To explain such a long-lasting mitigation in the disease progression, it has been proposed that *mdx* myofibers regenerate with a special efficiency. This feature could be related to the high levels of basic fibroblast growth factor (bFGF), and to the heightened sensitivity of satellite cells to this molecule detected in *mdx* muscles [2, 10, 11, 17]. However, comparative studies have shown that the regeneration efficiency of *mdx* muscles is substantially similar to that of control mice [15, 32].

It seems obvious that, at variance with DMD muscles, those of *mdx* are not only able to regenerate, but also to express after regeneration some factors protecting them from further necrosis.

A possible explanation is that an acquired resistance is supported by an incomplete maturation of the regenerated fibers. Indeed, unlike in humans, *mdx* regenerated fibers indefinitely retain centrally located nuclei, that are otherwise a feature of fetal myotubes [20]. Therefore, the prevalence of centronucleation is a reliable cumulative index of all prior myofiber necrosis [20]. The progressive increase of this prevalence with aging and the parallel decrease of fiber necrosis [6, 36] indicate that *mdx* regenerated fibers are for some reason less susceptible than the original ones.

It is also known that, in several genetic diseases of skeletal muscle, the deleterious effects of abnormal gene expression are suppressed in immature muscle fibers [18]. The molecular basis of this phenomenon may be in the expression of fetal isoforms of the lacking proteins, or in the reappearance of some characteristics of immaturity.

Among the latter, a reduced calibre of regenerated muscle fibers might enable *mdx* mice to overcome the lack of dystrophin. Indeed, a low ratio between cellular volume and external surface is peculiar of muscle fiber populations which are not involved in the process of necrosis. This applies to extraocular muscles, that are known to be spared in *mdx* mice [20]. Besides, necrosis has rarely been observed in skeletal muscles of *mdx* mice younger than 15 days of age, or in muscles which were experimentally denervated [20]. All these cases have in common a small diameter of fibers (less than 20 μm). Moreover, fibers of small diameter seem to have a better destiny not only in the *mdx* mouse, but also in the cardiomyopathic hamster [19] and in rare cases of DMD with dwarfism [18]. These data suggest that there are some advantages for small caliber myofibers that, having a smaller contractile mass, generate less strain per sarcolemmal surface unit.

As an alternative, or in addition to an incomplete maturation, other factors such as changes in biochemical and physiological properties might rescue regenerated *mdx* fibers. Indeed, changes in the prevalence of muscle fiber types have been reported during the life span of *mdx* mice. However, these studies have shown contrasting results [6, 7, 25, 31].

With this in mind, the aim of this study was to search for factors that could make *mdx* muscle fibers resistant to damage after regeneration. According to this, we addressed the following questions: 1) is there in limb skeletal muscles, as in extraocular muscles, a correlation between fiber calibre and susceptibility to necrosis? 2) are there modifications of metabolic and functional characteristics in these muscles? 3) is there an arrested development of regenerated myofibers, with permanent expression of fetal features? In order to clarify these points we studied the following parameters in *mdx* mice at three different ages, corresponding to three phases of the disease. 1) the mean cross-sectional area (CSA) of original and regenerated muscle fibers; these two categories were defined by the peripheral or central position of myonuclei, respectively [6]; 2) the prevalence of fibers type I, II and IIc by the ATPase reaction, and 3) the correlation between di-

ameter of regenerated fibers and their staining intensity by immunohistochemical reaction for developmental myosin heavy chain (d-MHC).

Materials and Methods

Stocks of *mdx* and C57BL/10ScSn normal mice were bred in our animal house. The animals were maintained in routine conditions on a standard commercial diet.

The study has been performed in groups of male *mdx* and normal mice at different ages: 1) at 17 days, when myofibers are structurally intact [26]; 2) at 60 days, when necrosis has involved large groups of fibers, and numerous regenerated myofibers are present; 3) at 180 days, when a majority of *mdx* fibers are regenerated. Six animals from each group were injected with an overdose of Pentothal i.p., and the gastrocnemius muscles were dissected and immediately frozen in liquid nitrogen-cooled isopentane. Serial transverse cryostat sections of the whole muscles were: a) stained with hematoxylin-eosin for morphometric analysis; b) stained with the histochemical method for myosin ATPase; c) immunostained with an antibody recognizing d-MHC.

Morphometric analysis has been performed with an automatic image analysis system (Leica Germany), separately recording the CSA of the original and regenerated fibers (at least 500 per animal) in randomly selected fields from cross sections of the whole muscle. Necrotic fibers, when present, were discarded. For each group of animals, distribution histograms of muscle fiber area were obtained.

For ATPase histochemistry, we observed in preliminary experiments that, in sections preincubated at pH 4.1, mouse muscle fibers can easily be classified in three groups, i.e.: type I fibers (dark staining), type IIa, IIb and IIx fibers (no staining), type IIc fibers (light staining). Therefore, we adopted this technique in order to detect three fiber types with a single reaction. The prevalence of fibers belonging to these three groups were calculated in the deep portion of gastrocnemius, where all fiber types are represented [3]. In *mdx* muscles, the percentage of type IIc fibers at different ages was compared to that of centronucleated fibers in order to have an index of the regenerated fibers showing immaturity.

Statistical analysis of the mean CSA of original and regenerated fibers, and of the prevalence of each histochemical fiber type in the different groups of animals was performed by the Student's t-Test (two-sample assuming equal variances).

For immunohistochemistry, unfixed cryostat sections were preincubated in normal rat serum (30 min.), incubated in a monoclonal antibody (IgG1 fraction) to d-MHC (Novocastra, UK) 1:20 in PBS for 60 min., followed by a biotinylated rat monoclonal antibody to mouse IgG1 (Zymed, USA) and by the avidin-biotin-peroxidase technique (Vector, USA). Muscle fibers expressing d-MHC were examined, and classified with an arbitrary staining intensity scale as: +++ (dark), ++ (intermediate), and + (light). The mean diameter (calculated after measuring the

maximum and minimum diameters on microphotographic prints) of every d-MHC positive fiber, and its intensity of staining were recorded and statistically matched for linear regression.

Results

Morphometric analysis

At 17 days, virtually all myofibers had peripheral nuclei, both in *mdx* and control mice. There was no difference in the mean CSA of original muscle fibers between the two groups of animals (Fig. 1 A, B).

In 60-day-old *mdx*, original myofibers were 60% of total;

the remainder 40% of all non-necrotic fibers were regenerated fibers. The mean CSA of original and regenerated *mdx* fibers were 35% and 43% lower, respectively, than CSA of control mouse fibers (Fig. 1 C, D).

At the age of 180 days, the amount of regenerated fibers in *mdx* increased to 60% of non-necrotic fibers, while original fibers declined to 40%. The mean CSA of original *mdx* fibers was 55% lower, whereas the CSA of regenerated *mdx* fibers was 5.5% higher (n.s.), as compared to control fibers. It should be considered that although the latter value was not significant, fibers of more than 5000 μm^2 were 15% of the total of regenerated fibers in *mdx*,

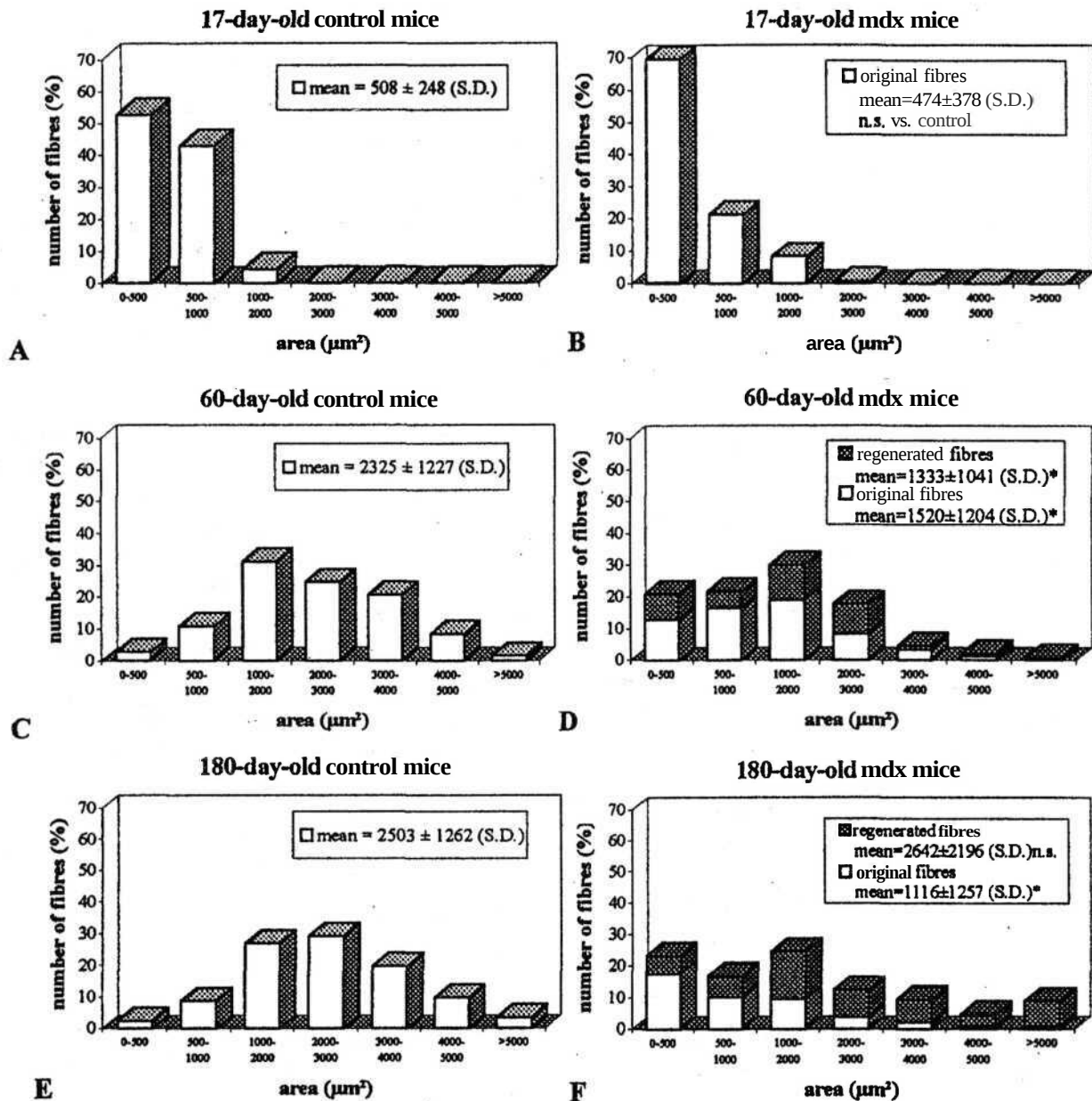


Figure 1. Fiber size distribution in the gastrocnemius of control (A, C, E) and *mdx* (B, D, F) mice at different ages. Note the progressive disappearance of large original fibers (D, F), substituted in part by large regenerated fibers (F), in *mdx*. ($n = 5$; S.D. = standard deviation; * = $p < 0.05$ at Student's *t*-test versus age-matched control; n.s. = non significant).

while being only 5% of all control mouse fibers (Fig. 1 E, F).

ATPase histochemistry

The prevalence of muscle fiber types by ATPase reaction is summarized in Table 1.

At 17 days, there were no differences between *mdx* and control mice in the prevalence of the three groups of muscle fibers.

By 60 days, type I and type II (a, b, x) fibers were equally distributed in *mdx* and controls. On the other hand, type IIc fibers were significantly increased in *mdx* with a four-fold increment as compared to controls. ($p < 0.05$)

In 180 day-old *mdx* mice, type I fibers were slightly increased versus controls, but this difference was not significant. The prevalence of type II (a, b, x) fibers was significantly decreased by about 9% in *mdx*. ($p < 0.05$) Finally, the prevalence of type IIc fibers in *mdx* was almost seven-fold that in control mice. ($p < 0.05$)

Notwithstanding the significant increment of fibers staining as type IIc, observed in *mdx* muscles at 60 and 180 days, these fibers represented respectively 14% and 10% of all regenerated fibers.

Immunohistochemistry

In 17-day-old mice, both *mdx* and control, no reactivity for d-MHC was observed, even in fibers which stained as type IIc by ATPase histochemistry. Afterwards, immunoreactivity was detectable only in myotubes and in small regenerating myofibers of the *mdx* muscle, while it was absent in large regenerated fibers. Indeed, according to our staining intensity scale, fibers expressing d-MHC at the highest level (+++) had a mean diameter of $3.3 \pm 1.3 \mu\text{m}$, intermediate fibers (++) had a mean diameter of $5.4 \pm 1.9 \mu\text{m}$, and fibers with low expression (+) had a mean diameter of $6.9 \pm 1.9 \mu\text{m}$ (Fig. 2). The linear regression test assuming fiber diameter as a dependent variable and staining intensity as an independent variable was highly significant ($R = 0.6758$, $P = 0.000$).

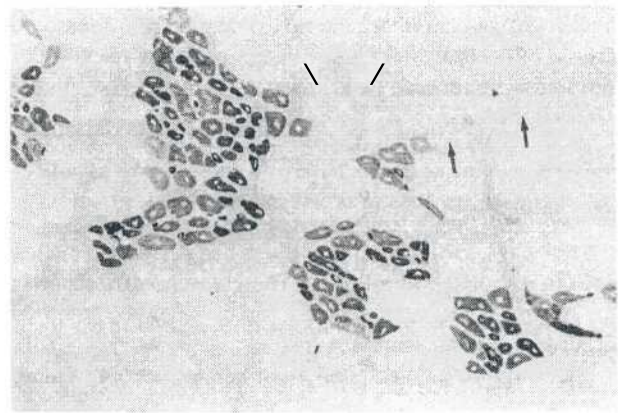


Figure 2. Immunohistochemistry for d-MHC in gastrocnemius from a 60 day-old *mdx* mouse. Note that immunoreactivity is greatest in small regenerating fibers, while it is low in larger regenerating fibers and absent in mature regenerated fibers, recognizable by central nuclei (arrows). $\times 200$.

Discussion

This study shows that the lack of repeated necrosis in *mdx* muscles is not due to an arrested maturation or to histochemical changes of regenerated fibers. Thus, the viability of these fibers has to be achieved through other and still unknown mechanisms.

Morphometry

The percentages of regenerated fibers detected in *mdx* gastrocnemius at 60 and 180 days are consistent with those observed by Coulton et al in the *mdx* soleus [7]. While other authors reported higher values for the same ages, these variances may be ascribed to the different muscles studied. [6, 20, 23, 33]. Moreover, since transverse sections not always pass through central nuclei, a certain underestimation of their value is possible [36]. However, the relevant number of original fibers we detected in 180 day-old *mdx* can explain why a low-grade, persistent necrosis has been observed in mice beyond this age [21, 33].

Table 1. Percentages of type I, II (a, b, x), and IIc fibres in control and *mdx* mice at different ages.

	I	II	IIc
<i>mdx</i> 17 days (n = 5)	6.7 ± 2.3 (n.s.)	87.6 ± 2.5 (n.s.)	5.7 ± 2.7 (n.s.)
control 17 days (n = 6)	9.1 ± 2.2	86.2 ± 1.9	4.7 ± 2.6
<i>mdx</i> 60 days (n = 5)	8.0 ± 1.7 (n.s.)	86.0 ± 4.5 (n.s.)	6.0 ± 3.6*
control 60 days (n = 6)	7.3 ± 3.1	91.3 ± 3.5	1.4 ± 1.1
<i>mdx</i> 180 days (n = 4)	12.2 ± 2.8 (n.s.)	81.0 ± 4.1*	6.8 ± 4.5*
control 180 days (n = 6)	9.7 ± 4.3	89.3 ± 4.0	1.0 ± 0.8

Values are means ± S.D.. * $p < 0.05$ at Student's *t*-test versus matched control. n.s. = non significant.

The results of morphometric analysis showed that, before the onset of necrosis, the mean CSA of *mdx* myofibers is comparable to that of control mice. However, with progression of necrosis (at 2 months, and more markedly at 6 months), the largest among original fibers tend to disappear from the histogram and there is a favourable selection of small fibers. This indicates that small caliber fibers of *mdx* undergo necrosis late in the life of these mice (after 6 months).

This implies a clear advantage for small myofibers and is in accordance with the reported resistance of small extraocular muscle fibers and of denervated skeletal muscle fibers in the *mdx* mouse [20]. The latter condition, however, induces disuse as well as fiber atrophy.

Our separate analysis of regenerated fibers showed a completely different behaviour of these fibers, as compared to the original ones. Indeed, at 60 days, the distribution histogram of regenerated fibers is shifted toward low values, and almost all fibers lie in categories of CSA below 3000 μm^2 . This is due to the fact that, by this age, most regenerated fibers have not completed their growth. On the contrary, at 180 days, the histogram of regenerated fibers is shifted toward larger values, and the group of fibers exceeding 5000 μm^2 is larger than in control mice.

The tendency of many regenerated fibers to hypertrophy, as we have observed, indicates that a large calibre is not an unfavourable factor for these fibers as it is for the original ones.

Several authors have studied the morphometric characteristics of *mdx* muscles although, in most cases, they have not examined original and regenerated fibers separately. Therefore, when comparing the mean CSA or mean diameter of *mdx* myofibers to those of controls, they reported little or no variation [1, 7, 22, 25]. However, in the latter study, a significant overall trend of *mdx* fibers to be larger was detected.

In the only existing study in which the diameters of original and regenerated *mdx* myofibers were separately recorded, no significant differences between these categories or with control fibers were reported [4]. Surprisingly, this work failed to detect any change even in the overall frequency distribution of fiber diameter. On the contrary, most reports described an increased variation in fiber size, with the presence of very small and very large fibers in the *mdx* [1, 7, 17, 22, 36 and present data].

In conclusion, our data are important in indicating that *mdx* hypertrophic fibers are not surviving original fibers, but fully regenerated fibers.

Histochemistry

Our results show that, in the pre-necrotic phase, there are no differences between *mdx* and control mice as to the prevalence of histochemical myofiber types. In particular, the low percentage of type IIc fibers indicates that, by this age, *mdx* myofibers are well differentiated, as also those of control mice. However, an increased prevalence of fibers staining as type IIc is evident in *mdx* at 60 and 180

days. Nevertheless, these fibers represent only between 10 and 14% of all regenerated fibers at these time points. This means that only a minority of regenerated fibers, at a given time, shows histochemical immaturity, which therefore seems to be a temporary condition of all regenerated fibers. Similar values of type IIc fiber prevalence have been reported for these ages in *mdx* soleus by Pastoret and Sebillé [31], while DMD muscles show higher values [30].

In 180 days old *mdx*, there is a significant although not dramatic reduction in the percentage of type II (a, b, x) fibers. On the other hand, type I fibers show a trend to increase, but the difference with controls is not significant.

These results seem to indicate that a moderate shift from type II to type IIc and type I fibers takes place in the muscle of adult *mdx*. Such a shift, however, is far too limited to imply important changes in the functional and metabolic characteristics of these muscles.

A decrease of type II fibers in the soleus of *mdx* mice of the age 18 weeks was reported also by Pastoret and Sebillé [31]. Studying the same muscle, Carnwath and Shotton [6] showed a more pronounced shift from type II to type I fibers, with a rise of type I from 28% at 3 weeks, to 58% at 26 weeks. Louboutin et al. [22] reported a similar trend. Whereas, Marshall et al. [25] reported a markedly lower proportion of type I fibers in *mdx* soleus (< 30%) than in control (> 70%), at all ages. Such important discrepancies in data relative to the same muscle at the same age are clearly unexplained. Finally, studying the fast contracting EDL muscle, Louboutin et al. found no difference in the proportion of type I, IIa, or IIb fibers between *mdx* and control mice at different ages [23].

Therefore, the majority of these studies suggest that the original myofibers of *mdx* mice share with DMD muscle fibers a preferential degeneration of fast contracting fibers [28, 37]. However, at variance with humans, in adult mice these fibers show a decreased susceptibility and become hypertrophic [36].

It should be considered that ATPase histochemistry in the mouse does not allow to differentiate clearly between type IIa and type IIb muscle fibers [7], and type IIx, that are abundant in fast-twitch muscle of the mouse, are only detectable by using specific antibodies [14, 38]. Therefore, we cannot rule out that shifts between these groups take place. Indeed a reciprocal conversion between IIb and IIx fibers is considered a normal phenomenon in mouse muscles [38]. However, such shifts would not alter the ratio between fast-twitch and slow-twitch fibers, necessary to induce major changes in the physiological characteristics of these muscles.

Immunohistochemistry

The antibody we used for detecting d-MHC recognizes a MHC present during the embryonic and neonatal period in the development of skeletal muscle and during regeneration of muscle fibers [9].

In 17 day-old animals, either controls or *mdx*, we did not detect positive fibers for d-MHC, although a certain number of fibers were classified as type IIc by ATPase

histochemistry. Type IIc fibers are generally considered immature but, at least in mice, mature fibers classified as type IIc have also been described [31]. On the contrary, in 60 and 180 day old *mdx* mice, the foci of small immature regenerating fibers were of type IIc and most of them expressed d-MHC. However, linear regression analysis showed that the intensity of staining for d-MHC was inversely proportional to fiber diameter, being maximal in myotubes and gradually decreasing with fiber growth. It is noteworthy that large calibre regenerated fibers were devoid of d-MHC reactivity.

The presence of d-MHC reactive myofibers in adult *mdx* mice but not in 17 day-old mice indicates a *de novo* expression in regenerating fibers, rather than a permanent expression due to arrest in development. The same feature has been observed in DMD [37].

Our data are consistent with those of Di Mario et al that, using an antibody for embryonic myosin, detected expression only in 8-10% of *mdx* myofibers, without specifying their degree of maturation [12]. These values are markedly lower than those reported in DMD [37]. Moreover, our morphometric analysis enables us to infer that the expression of d-MHC is not a permanent feature of a population of regenerated fibers, that for some reason retains properties of fetal fibers. On the contrary, this is characteristic of and limited to myofibers in the first stages of regeneration. A similar trend of expression in *mdx* regenerating fibers has been reported for the neural cell adhesion molecule (N-CAM), which is a developmentally regulated molecule [13].

Altogether, our results suggest that, in *mdx* original muscle fibers, at least two factors exist that confer partial resistance to necrosis: these are a small caliber and type I at histochemical classification. Surprisingly, these factors are not necessary for regenerated fibers to survive without dystrophin. Indeed, these fibers mature, becoming even larger than the original ones, and they remain prevalently of type II in the gastrocnemius, yet they do not seem to undergo further cycles of necrosis.

These results, and the abrupt onset of myofiber degeneration at the age of three weeks, suggest that in *mdx* mice the deleterious consequences of dystrophin defect can be triggered or mitigated by additional factors that seem to be differentially expressed during development and regeneration.

One of the mitigating factors may be represented by centronucleation, that may favour a greater transcriptional activation [34]. This is consistent with the increased total RNA and protein content observed in *mdx* limb muscles [24]. Moreover, in hypertrophic fibers, an internal position can reduce the distance between a nucleus and the periphery of its domain of influence in the sarcoplasm, facilitating nuclear control on cellular trophism and metabolism. Interestingly, in the *mdx* diaphragm, which shows early dystrophic features, the percentage of centronucleated fibers is markedly lower than in limb muscles [4, 22].

In conclusion, *mdx* mice can rely on compensatory mechanisms that, albeit defective in the diaphragm and less effective during senescence, allow them to survive without motor impairment for most of their life. The mechanisms by which *mdx* muscles restore an adequate function and become stable in the absence of dystrophin may, when clarified, provide important clues for planning effective non-genetic therapeutic approaches to DMD.

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