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EXPERIMENTAL REGENERATION IN CANINE MUSCULAR DYSTROPHY—1. IMMUNOCYTOCHEMICAL EVALUATION OF DYSTROPHIN AND β-SPECTRIN EXPRESSION

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Abstract—The expression of dystrophin and β-spectrin was examined from 1 to 56 days in regenerating muscle fibres in normal and dystrophic dogs, following necrosis induced by the venom of *Notechis scutatis*. Normal and dystrophic dog muscle regenerated at an equal rate and new myotubes were present in both at the periphery of necrotic fibres by 3 days. In normal dogs dystrophin was detected in the sarcoplasm of the regenerating fibres by 3 days and was localized to the plasma membrane by 4 days. The localization of dystrophin is independent of β-spectrin and was detected before β-spectrin, which was not observed until 5–6 days. Normal peripheral labelling of both was restored by 14 days in normal dogs. Normal β-spectrin labelling of regenerating dystrophic fibres was also restored by 14 days and is not dependent on the presence of dystrophin in dystrophic dogs. A proportion of regenerating fibres in normal and dystrophic dogs showed weak immunolabelling of β-spectrin prior to 14 days. This is a feature of immature muscle fibres. Antibodies to different domains of dystrophin bound to the periphery and sarcoplasm of regenerating fibres in dystrophic dogs, particularly during the first 7 days of regeneration, but the fluorescence was less intense than in normal dogs. Weak labelling with antibodies corresponding to the C-terminus of the rod domain of dystrophin persisted on dystrophic regenerating fibres up to 21 days. This may relate to developmental isoforms of dystrophin.

Key words: Dystrophin, β-spectrin, regeneration, dystrophic dog, muscular dystrophy.

INTRODUCTION

Canine X-linked muscular dystrophy (CXMD) has many features in common with Duchenne muscular dystrophy (DMD) [1–3] and is an ideal model in which to study the disease process and to develop potential therapeutic techniques. Affected *xmd* dogs have a progressive myopathy that shows similar pathological features to those found in DMD [2, 3] and both skeletal and cardiac muscle lack expression of dystrophin, the defective gene product responsible for DMD [1]. The lack of dystrophin has recently been shown to be caused by an error in RNA processing and a splice-site mutation [4].

Myoblast transfer has been proposed as a possible therapy for DMD and experiments using this technique have been performed in humans [5] and the *mdx* mouse [6]. It is essential, however, that all aspects of myoblast transfer are thoroughly investigated before the technique can be recommended for therapeutic use in humans. A proportion of the dystrophin-positive fibres present after the injection of normal myoblasts develop from myotubes derived from the fusion of donor cells [6]. The formation of these new fibres resembles embryonic myogenesis and regeneration but it is not known if they develop, mature and function normally in dystrophic muscle. An understanding of the pattern of regeneration of normal and dystrophic muscle will therefore provide a reference for assessing the development and growth of the new fibres formed from transplanted myoblasts. We have initiated a detailed morphological study of skeletal muscle regeneration in normal and dystrophic

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dogs to provide this reference data for the assessment of myoblast transfer therapy in the dog model. In addition, the study was designed to provide a greater understanding of the development of normal and dystrophic muscle and to identify any possible early developmental defects in dystrophic muscle.

The major criterion for the success of myoblast transfer in the *mdx* mouse has been the detection of dystrophin [6, 7] but little is known about the time-course of dystrophin expression during regeneration *in vivo* or its relationship to developmentally regulated muscle proteins. There is increasing evidence that there are embryonic isoforms of dystrophin [8–10], and in muscle from foetuses at high risk for DMD some binding of dystrophin antibodies can be detected at certain stages [11]. The assessment of myoblast transfer is therefore dependent on a full understanding of the immunostaining patterns with dystrophin antibodies in both normal and dystrophic developing and regenerating muscle.

We report here an evaluation of dystrophin immunostaining in regenerating normal and dystrophic dog muscle following damage induced by the venom of the tiger snake *Notechis scutatus*. Certain snake venoms cause marked muscle fibre necrosis and have proved useful for studies of muscle regeneration [12]. Previous work with the venom of *Notechis scutatus* has shown that muscle regeneration occurs within a few days of induced necrosis and that functional fibres are restored [13, 14]. This model can therefore be used to examine the expression and inter-relationships of several muscle proteins during the maturational process.

In this study we have compared the expression of dystrophin with that of β-spectrin, and a cytoskeletal function has been suggested for both dystrophin [15] and β-spectrin [16]. The localization of dystrophin and β-spectrin is also similar and both are detected at the sarcolemma [17, 18]. A comparison of these two proteins after toxin-induced damage will provide information on the breakdown of the sarcolemmal membrane during the necrotic phase and on the subsequent development of dystrophin and β-spectrin during regeneration, and should identify any defects relating to an absence of dystrophin.

METHODS

Necrosis was induced by the injection of 1 μg of the venom of the tiger snake, *Notechis scutatus*, dissolved in isotonic saline. Venom was injected into the surgically exposed superficial layers of the right biceps femoris of 6-month-old normal and dystrophic dogs, under anaesthesia. The injected site was marked with sutures and open biopsies taken each day from 1 to 8 days and at 10, 14, 21, 28, 42 and 56 days thereafter. A maximum of four biopsies over the 56 day period were taken from each of six normal and six dystrophic dogs. Control samples from the left, uninjected biceps femoris were also taken at each time point.

All samples were mounted in OCT on cork discs and frozen in isopentane cooled in liquid nitrogen. Unfixed serial cryostat sections (6 μm) were immunolabelled with four antibodies against different regions of dystrophin: H12 and P6 rabbit polyclonal antibodies raised in our laboratory [19] to fusion proteins derived from the C-terminal region of the rod domain (amino acids 2604–3024 and 2814–3028, respectively); Dy4/6D3 and Dy8/6C5 mouse monoclonal antibodies raised to the mid-rod region and the last 17 C-terminal amino acids, respectively [20, 21] (commercially known as Dys1 and Dys2). The localization of these antibodies was compared with that of a mouse monoclonal antibody to human erythrocyte β-spectrin (56A). Primary antibodies were applied for 30 min (H12 1 : 1200; P6 1: 800; Dy4, Dy8 and 56A were used undiluted). Sections were washed and labelled with an appropriate biotinylated secondary antibody for 30 min (1 : 200, Amersham, U.K.) and after further washing, antibodies were visualized with streptavidin conjugated to Texas Red (1 : 200, Amersham, U.K.) for 15 min and mounted in UVinert (BDH, U.K.). All dilutions and washings were made with phosphate buffered saline pH 7.2. Serial areas were identified and photographed.

Control sections were labelled without the primary antibody and with pre-immune serum from the rabbits in which the polyclonal antibodies were raised. Serial sections to those immunolabelled were also stained histologically with haematoxylin and eosin.

RESULTS

Controls

All control sections labelled without primary antibody were negative except for autofluorescence, including necrotic fibres. With pre-immune serum the muscle fibres (including regenerating

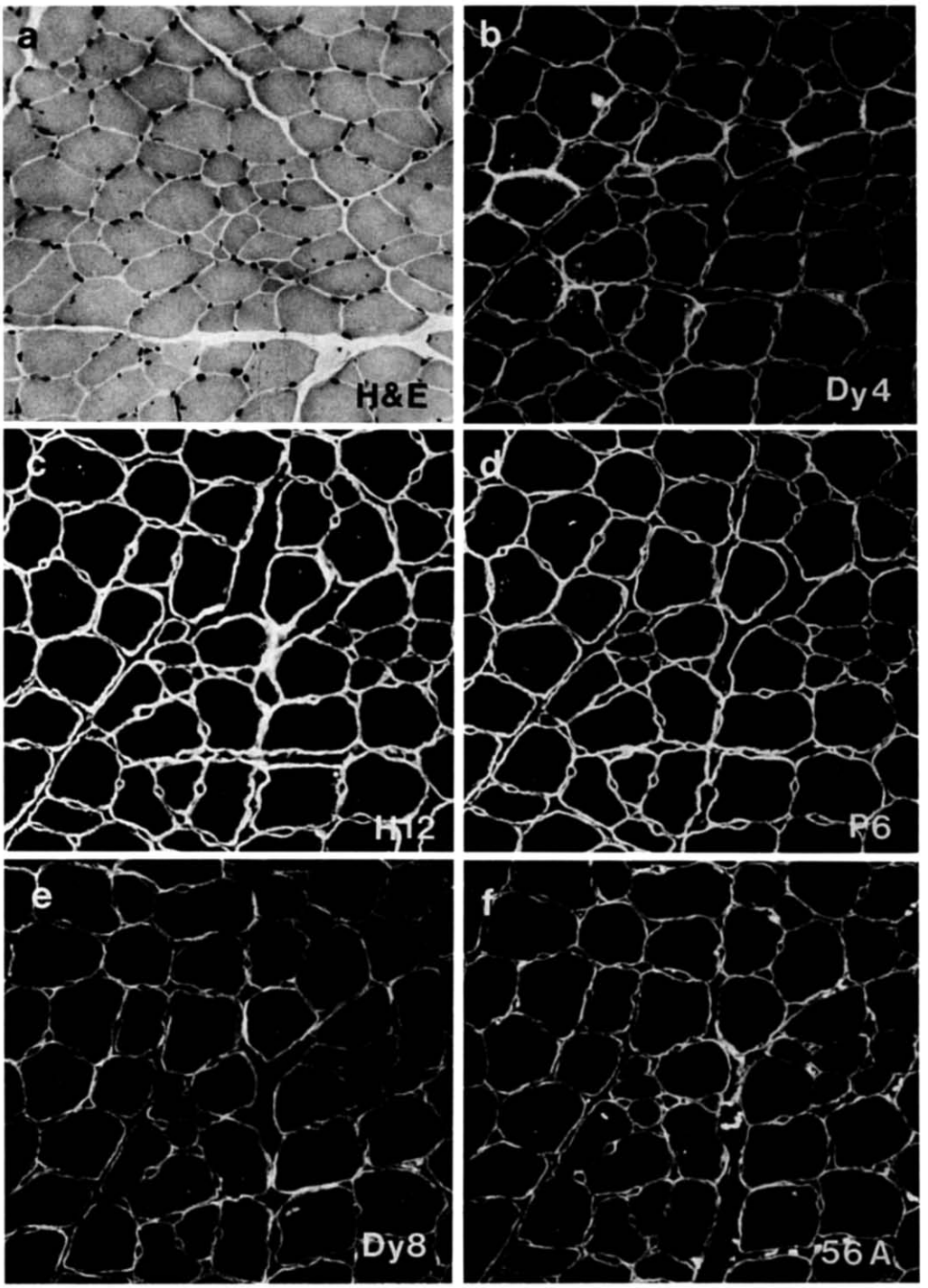

Fig. 1. Biceps femoris from a normal 6-month-old dog stained with: (a) haematoxylin and eosin to show the normal fibre size variation; (b)–(e) serial areas immunolabelled with antibodies to dystrophin, Dy4, H12, P6, Dy8 respectively; (f) β-spectrin, 56A, showing uniform sarcolemmal labelling with all antibodies. × 195.

fibres) were negative but discrete interstitial areas were labelled. These non-specific areas were present to varying degrees in normal and dystrophic samples and with the monoclonal dystrophin antibodies (see Figs 4 and 7). The pre-immune rabbit serum also bound non-specifically to necrotic fibres, as did the monoclonal dystrophin antibodies.

Normal dogs

Normal, uninjected muscle from 6-month-old dogs showed fibres ranging from 15 to 85 μm in

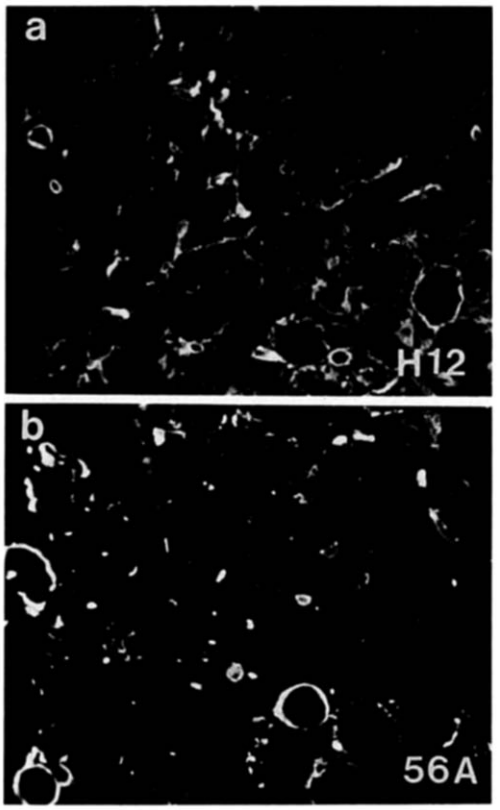

Fig. 2. Normal dog biceps femoris 1 day post-injection of snake venom immunolabelled with antibodies to (a) dystrophin, H12 and (b) β-spectrin showing the loss of sarcolemma from most fibres. × 195.

diameter. The periphery of all fibres was uniformly labelled with the four antibodies to dystrophin and to β-spectrin and there was no sarcoplasmic staining (Fig. 1).

1–3 *days post-injection.* Twenty-four hours after the injection of snake venom, fibres in the injected area were pale with haematoxylin and eosin and the myofibrils disrupted. Dystrophin was only detected on a few fibres and this was often discontinuous. Similarly, β-spectrin immunostaining was grossly disrupted and was visible on very few fibres (Fig. 2).

Two days after injection the necrotic zone appeared very cellular and no specific immunostaining for dystrophin or spectrin was seen (Fig. 3). At the edge, and occasionally within the necrotic zone, fibres with normal immunolabelling of dystrophin and β-spectrin were seen. These were non-necrotic fibres that had escaped the effects of the toxin and were also identified with haematoxylin and eosin staining (Fig. 3).

By 3 days, regenerating myotubes were seen as a cuff at the periphery of necrotic fibres and sarcoplasmic traces of dystrophin were detectable

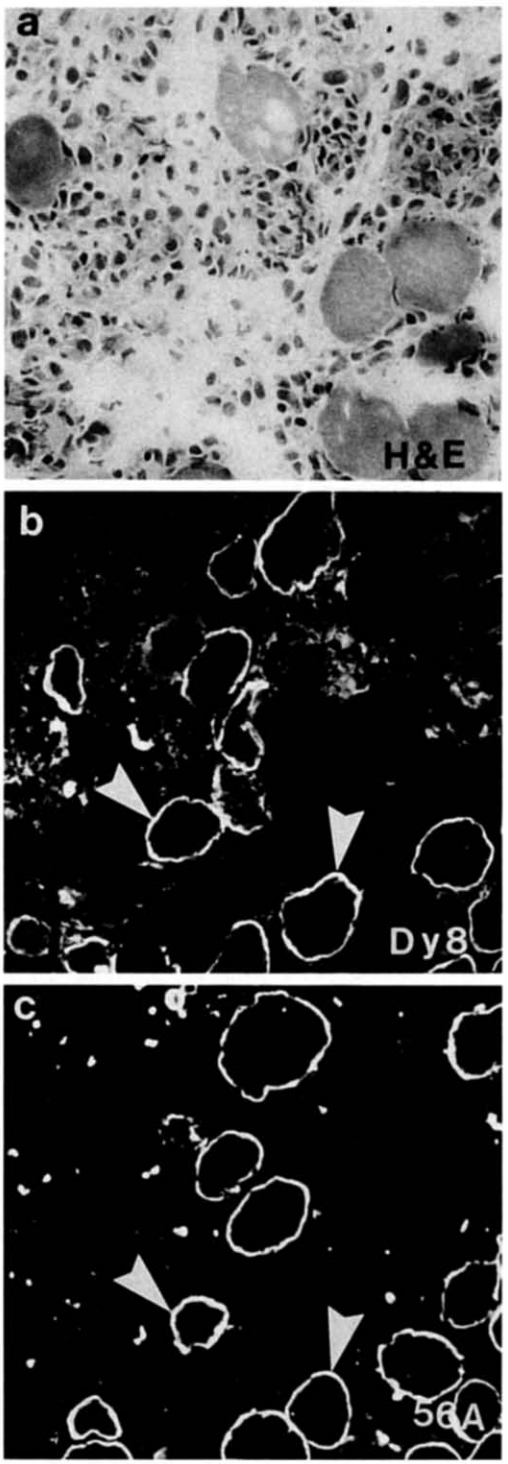

Fig. 3. Normal dog biceps femoris 2 days post-injection of snake venom, stained with: (a) haematoxylin and eosin; serial areas immunolabelled with antibodies to (b) dystrophin, Dy8, (c) β-spectrin. Note the cellular appearance of the necrotic zone which lacks dystrophin and β-spectrin, and the fibres that still express dystrophin and β-spectrin (arrows) and have escaped the effects of the venom. × 195.

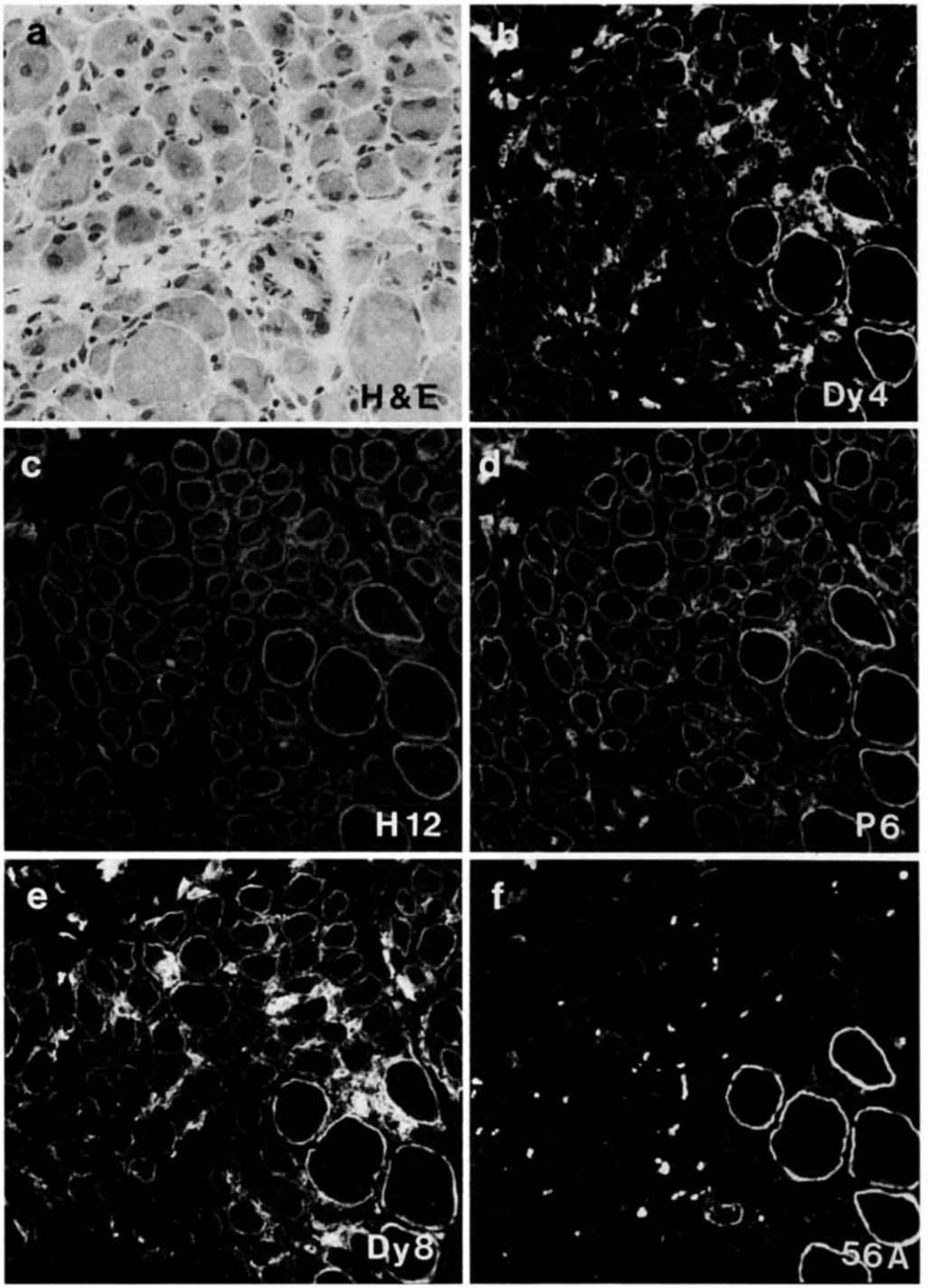

Fig. 4. Normal dog biceps femoris 5 days post-injection of snake venom, stained with: (a) haematoxylin and eosin showing basophilic fibres with peripheral nuclei; (b)–(d) serial areas immunolabelled with antibodies to dystrophin, Dy4, H12, P6, Dy8 respectively; (f) β-spectrin. Note the peripheral localization of dystrophin but the very weak labelling of β-spectrin of the regenerating fibres. Labelling is weaker with Dy4. The large fibres expressing dystrophin and β-spectrin are fibres that have escaped the effects of the venom. Labelled areas between fibres are non-specific. × 195.

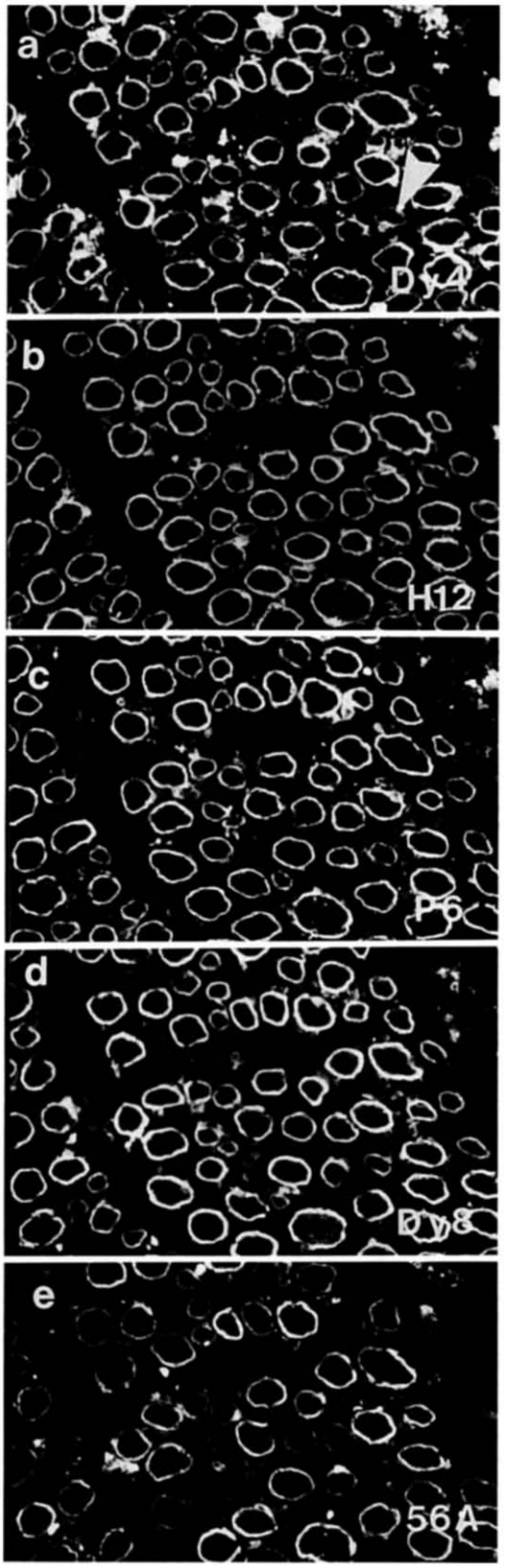

Fig. 5. Serial sections of normal dog biceps femoris 6 days post-injection of snake venom immunostained with antibodies to: (a)–(d) dystrophin, Dy4, H12, P6, Dy8, respectively; (e) β-spectrin, showing uniform labelling of most regenerating fibres with each dystrophin antibody but differential staining with β-spectrin. Some smaller fibres are less intensely labelled with Dy4 (arrow). × 195.

in these areas with H12, P6 and Dy4. No β-spectrin was detected in these myotubes. These areas were identified as regenerating by the presence of desmin, N-CAM and neonatal myosin (data not shown). In all subsequent samples the regenerating fibres were identified by their uniform expression of neonatal myosin.

4–6 *days post-injection.* By 4–5 days post-injection well-defined basophilic, regenerating fibres could be seen and the intensity of dystrophin staining had increased with all antibodies. It was particularly marked with H12 and P6. Peripheral staining was continuous and with H12 traces of sarcoplasmic staining were present. At 4–5 days β-spectrin was difficult to detect and staining was very weak (Fig. 4) but by 6 days labelling was more intense with variability between different fibres (Fig. 5). The smaller fibres were usually less intensely stained than larger ones. Dystrophin labelling was uniform and intense by 6 days but a little variability was seen with Dy4 and Dy8, with the smaller fibres sometimes more weakly stained than the larger ones (Fig. 5).

7–56 *days post-injection.* Dystrophin immunostaining was uniform on all fibres by 7 days post-injection but there was still some variability in β-spectrin staining at 7 and 10 days. By 14 days, and throughout the remaining test period, both dystrophin and β-spectrin labelling were indistinguishable from that of the normal controls (Fig. 6).

Dystrophic dogs

The uninjected contralateral muscles in dystrophic dogs showed no labelling of fibres with antibodies to dystrophin, except for regenerating fibres (identified by neonatal myosin) and occasional 'revertant' fibres which had normal labelling. Fibres were uniformly labelled with β-spectrin antibodies, with regenerating fibres usually weakly stained.

1–7 *days post-injection.* In dystrophic dogs the morphological pattern of regeneration was similar to normal with the appearance of well-defined basophilic, regenerating fibres by 4–5 days (Fig. 7). No specific membrane binding of the β-spectrin or Dy4, Dy8, and P6 dystrophin antibodies was seen at 4–5 days but H12 showed traces of labelling on most regenerating fibres, both peripherally and in the sarcoplasm (Fig. 7). By 7 days post-injection the labelling with H12 was more pronounced and traces with P6 and Dy8

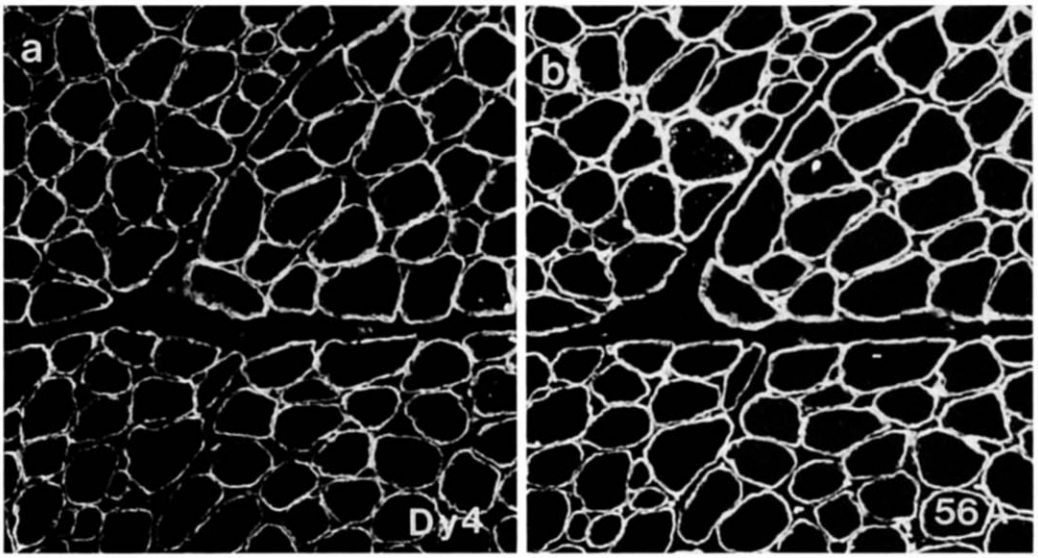

Fig. 6. Serial sections of normal dog biceps femoris 14 days post-injection of snake venom immunolabelled with antibodies to: (a) dystrophin, Dy4; (b) β-spectrin, showing the restoration of the normal pattern. × 195.

(and occasionally Dy4) were also detected (Fig. 8). This labelling was in addition to the occasional revertant fibres observed (< 1%). β-Spectrin was detected on most fibres by 7 days but the smaller diameter fibres were often less intensely labelled (Fig. 8). Regenerating fibres were identified by their expression of neonatal myosin (data not shown).

10–14 *days post-injection.* By 10–14 days post-injection, labelling with Dy4 and Dy8 was confined to the revertant fibres but both H12 and P6 were detected on many other regenerating fibres (Fig. 9). This labelling was weak compared to normal dog muscle. β-Spectrin showed variability in intensity between fibres at 10 days but by 14 days most fibres were uniformly labelled (Fig. 10).

21–56 *days post-injection.* At 21 days post-injection, weak labelling with H12 was still observed but none was seen with the remaining dystrophin antibodies and β-spectrin was uniform. At later stages clusters of fibres were often labelled with P6 and Dy4 (Fig. 11). From 28 to 56 days variation in fibre size was more apparent with several small fibres being present. These were usually labelled with H12 and P6 and often weakly labelled for β-spectrin.

DISCUSSION

The results presented demonstrate that normal and dystrophic muscle from 6-month-old dogs have an equal potential to regenerate. Well-defined regenerating fibres were present in both normal and dystrophic dog muscle by 3–4 days showing that a lack of dystrophin does not impede the initial stages of regeneration. Whether further development of these into mature fibres is affected is currently being studied, and it is not yet known if the regenerative capacity of older or younger dogs is different. Age differences might explain the inability of regeneration to keep pace with the muscle loss in muscular dystrophy but the results obtained in this study show that the initiation and early stages of regeneration occur in a similar manner in normal and dystrophic muscle.

The rate of regeneration in dogs is rapid and similar to that found in rats [22] and humans [23]. After injection of snake venom there is a lag phase of about 24 h before morphological signs of damage are apparent. This is followed by necrosis of the affected fibres but retention of the basement membrane (Sewry *et al.* in preparation) and the formation of new fibres by 3–4 days. Using 1 μg of venom, a local, but extensive, area of fibres was damaged in dog muscle. In contrast, a similar quantity in rats causes the necrosis of most of a muscle [13]. In dogs, it was therefore necessary to identify the injected site with sutures. The regenerative zone was identified in sections by the uniformly small fibres, compared to the control muscle, and by the presence of neonatal myosin. The latter was not expressed in the uninjected muscle of the left limb. This criterion was less applicable in dystrophic dogs at later stages (42 and 56 days)

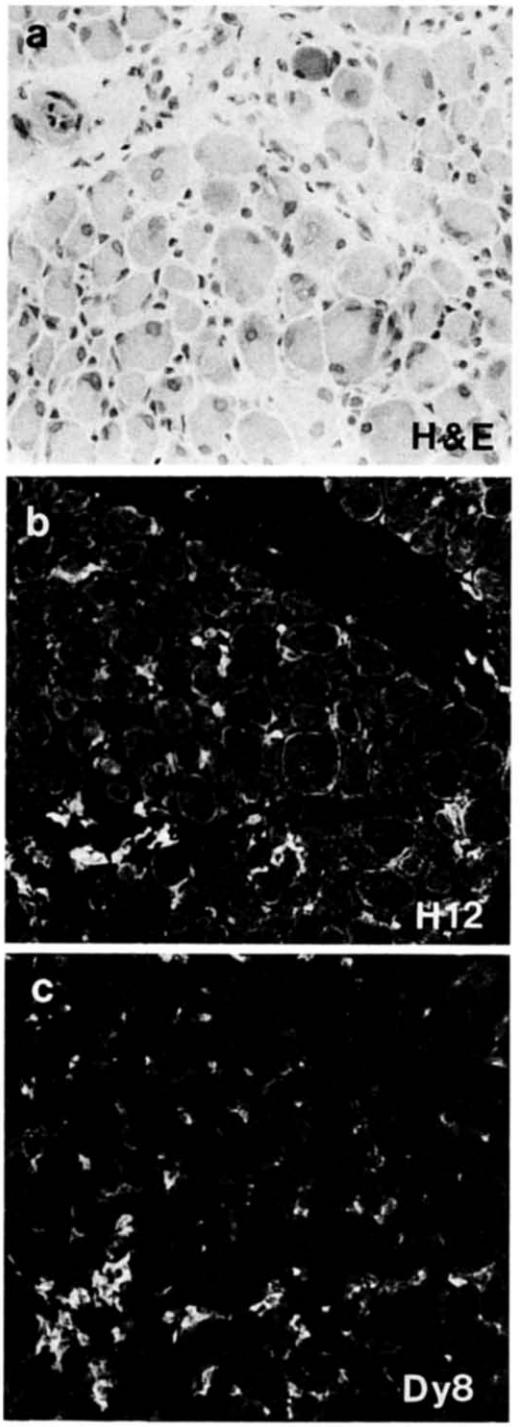

Fig. 7. Dystrophic dog biceps femoris 5 days post-injection of snake venom, stained with: (a) haematoxylin and eosin showing basophilic fibres with mainly peripheral nuclei and; serial sections immunostained with dystrophin antibodies (b) H12; (c) Dy8, showing peripheral and sarcoplasmic labelling of the fibres with H12 but not with Dy8. Non-specific labelling is present between the fibres with both antibodies. × 195.

because of the regeneration that inherently occurs.

The regenerating fibres in both normal and dystrophic dogs showed less variation in size than the uninjected control muscle, particularly at early stages. Quantitation of the fibre sizes is in progress. Variability in fibre size became more apparent by 14 days in normal dogs, and pathological differences in dystrophic dogs were observed as early as 5 days post-injection. Internal nuclei were not a feature of the normal regenerating muscle samples, but were more common in the dystrophic dogs. Most nuclei were peripherally located by 4–5 days, in contrast to those in rodents which remain centrally situated [24]. It is also of interest to note that the fascicular pattern of muscle fibre bundles was retained and that even in dystrophic dogs endomysial connective tissue was not excessive in the regenerating areas at early stages.

Dystrophin was first detected immunocytochemically in normal regenerating fibres at about 3 days but it was mainly cytoplasmic at this stage and not localized to the membrane. These small regenerating fibres resemble myotubes and form a cuff of cells at the periphery of the necrotic fibres. It is likely that dystrophin is present before this stage, as gene transcription is initiated as myoblasts differentiate into myotubes and low levels of dystrophin have been shown immunocytochemically on pre-fusion myoblasts *in vitro* [25]. This staining progressively increasing as fusion continues [25].

In normal dog muscle the appearance of dystrophin preceded that of β-spectrin, suggesting that they are independently regulated. Spectrin is believed to form part of the cytoskeleton in muscle, in a similar manner to that in erythrocytes [26], and it has been proposed that dystrophin may also be a cytoskeletal protein [15]. Recent studies have shown that dystrophin and β-spectrin colocalize at sarcolemmal domains overlying I bands and M lines and form a subsarcolemmal lattice [27]. It has been suggested that the lattice links the contractile apparatus to the sarcolemma and stabilizes the sarcolemma during contraction [27]. In the *mdx* mouse β-spectrin forms a lattice in the absence of dystrophin. Our results are consistent with these findings and show that β-spectrin can localize to the sarcolemma in the absence of dystrophin. In addition, they demonstrate that dystrophin does not require β-spectrin for anchorage to the sarcolemma, as dystrophin is detected before β-spectrin, and that dystrophin appears in advance of any major stress imposed on the fibres by contraction.

Differences in immunolabelling were seen with different dystrophin antibodies. In particular,

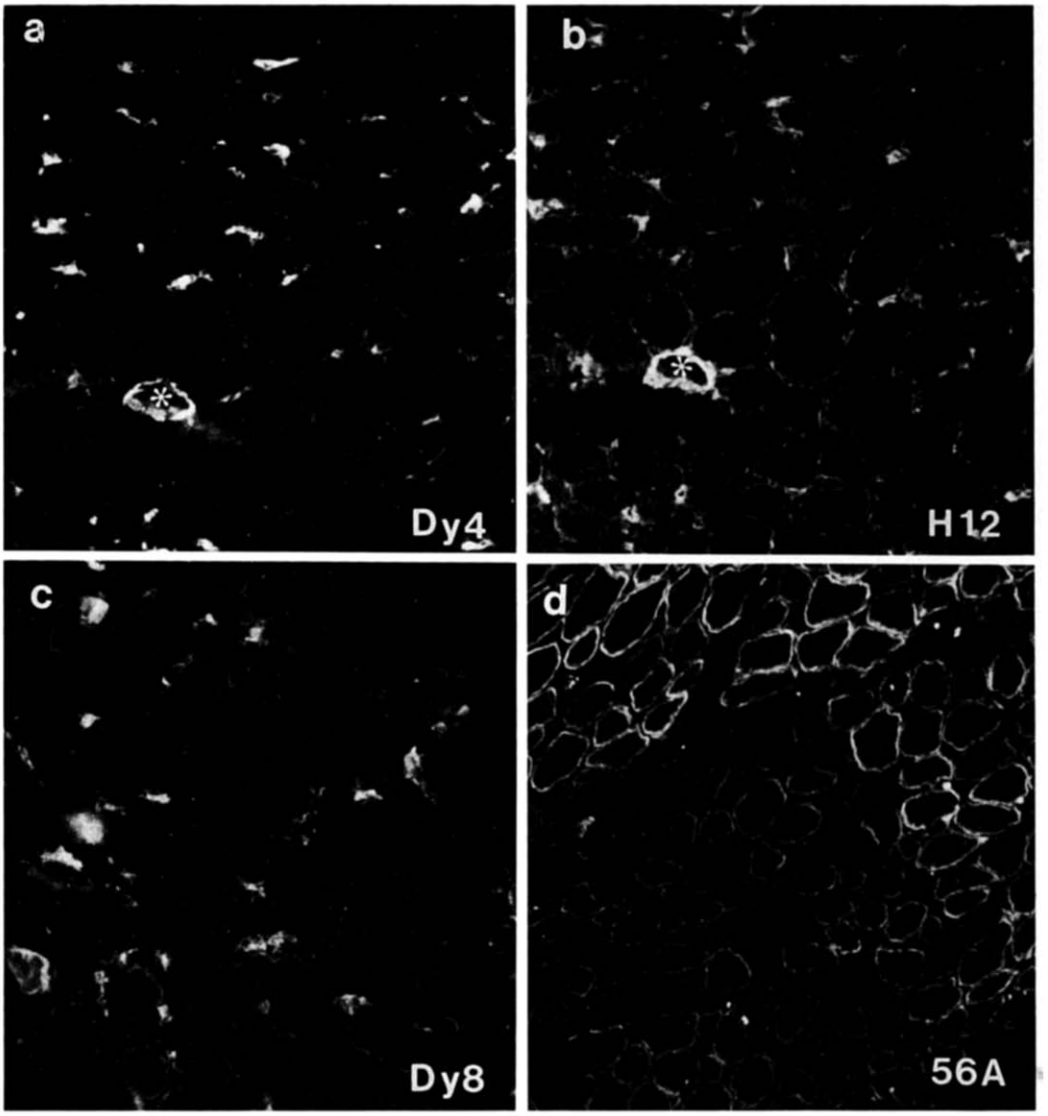

Fig. 8. Dystrophic dog biceps femoris 7 days post-injection of venom immunostained with antibodies to: (a)–(c) dystrophin, Dy4, H12, Dy8, respectively; (d) β-spectrin. Note the absence of staining on most regenerating fibres except the revertant fibre (*) with Dy4, in contrast to the labelling of most fibres including the revertant fibre with H12, and the weak labelling of most fibres with Dy8 (not serial). Some regenerating fibres are less intensely labelled with β-spectrin. × 195.

H12, and to a lesser extent P6, labelled the regenerating fibres from an early stage and the intensity of staining of all myotubes with Dy4 and Dy8 did not resemble that of controls until 10–14 days post-injection. Similar differences in staining have been observed in human foetal muscle, using the same panel of antibodies, where the results suggested that dystrophin is developmentally regulated and that isoforms may be present [11, 12]. There is now considerable evidence that the dystrophin locus can produce several different transcripts [8, 9, 28, 29] but any developmental role that these may have is not known. Although caution in interpreting the intensity of immunolabelling is always necessary and the affinity of antibodies can differ, our observations of regenerating fibres support the opinion that developmental isoforms of dystrophin can be expressed in skeletal muscle. The presence of developmental isoforms might also explain the binding of dystrophin antibodies to the regenerating fibres in dystrophic dogs. This finding was particularly apparent with the polyclonal antibodies (H12, P6), raised to the C-terminal region of the rod-repeat region, and was more evident at early stages and on clusters of small fibres at later stages. It is uncertain whether these small fibres in dystrophic dogs are atrophic or if they represent a secondary regeneration resulting from damage to the original regenerating fibres.

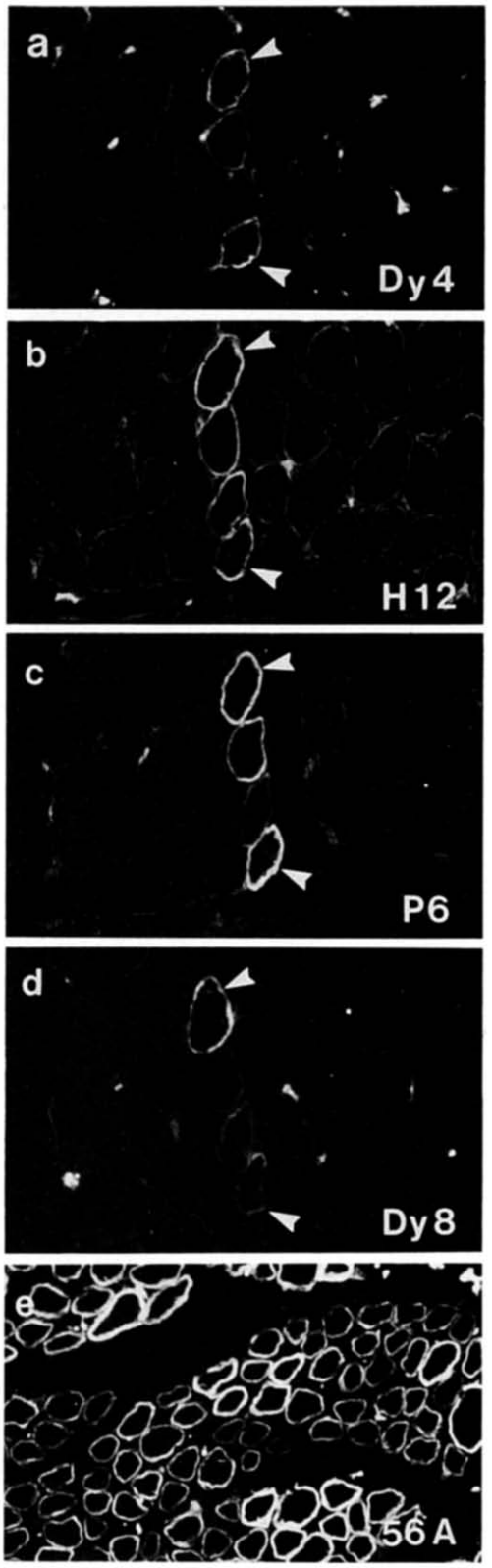

Fig. 9. Dystrophic dog biceps femoris 10 days post-injection of snake venom immunostained with antibodies to: (a)–(d) dystrophin, Dy4, H12, P6, Dy8, respectively; (e) β-spectrin. The monoclonals Dy4 and Dy8 only label revertant fibres (arrows) but H12 and P6 both show weak peripheral labelling of most regenerating fibres. Some fibres are still weakly labelled with antibodies to β-spectrin. × 195.

Although dystrophin antibodies labelled the regenerating fibres in dystrophic dogs, the intensity of fluorescence was less than in normal dogs and diminished with time. Dystrophic samples could easily be distinguished from normal ones and there is, therefore, unlikely to be any difficulty in assessing myoblast transfer experiments.

Binding of dystrophin antibodies to the regenerating fibres in dystrophic dogs might relate to the presence of the dystrophin homologue coded by human chromosome 6 and mouse chromosome 10, variously known as DMD-like protein (DMDL), dystrophin-related protein (DRP) or utrophin [30, 31]. Utrophin is abundant at the sarcolemma of regenerating fibres and in a variety of non-muscle tissues [32, 33] but the dystrophin antibodies used in our study do not detect a 400 kDa protein in foetal visceral tissues known to express utrophin [11] and in DMD some muscle fibres with pronounced binding of utrophin antibodies do not bind P6 or H12 (data not shown). There is thus no evidence for cross-reactivity with utrophin, as shown by current antibodies, and we have interpreted our data as representing labelling of dystrophin isoforms. The possibility of P6 and H12 dystrophin antibodies cross-reacting with an unknown, related protein, however, cannot be excluded.

Antibodies to β-spectrin showed variable labelling between regenerating fibres, with a population of fibres less intensely stained. These were prominent at 6 days in normal dogs and persisted to a diminishing degree until 10 days post-injection. In dystrophic dogs the weakly stained fibres could still be observed in some areas at 14 days but were apparent again at the later stages of 42 and 56 days. Endogenous regenerating fibres in human [34] and canine [35] dystrophic muscle show a similar reduction in staining intensity compared to normal fibres and the results presented here demonstrate that this relates to immaturity. This is supported by a similar observation in human foetal muscle [11]. If small fibres with weak expression of β-spectrin are immature, their presence at 42 and 56 days after injection suggests that additional bouts of regeneration occur in dystrophic muscle, as mentioned above. This implies that regenerating dystrophic fibres are also susceptible to the dystrophic process. This is contrary to the opinion of Karpati *et al.* that small calibre fibres are not affected by the disease process [36].

Our observations on the presence of dystrophin and β-spectrin 1 day after injection of venom demonstrate that both proteins are lost

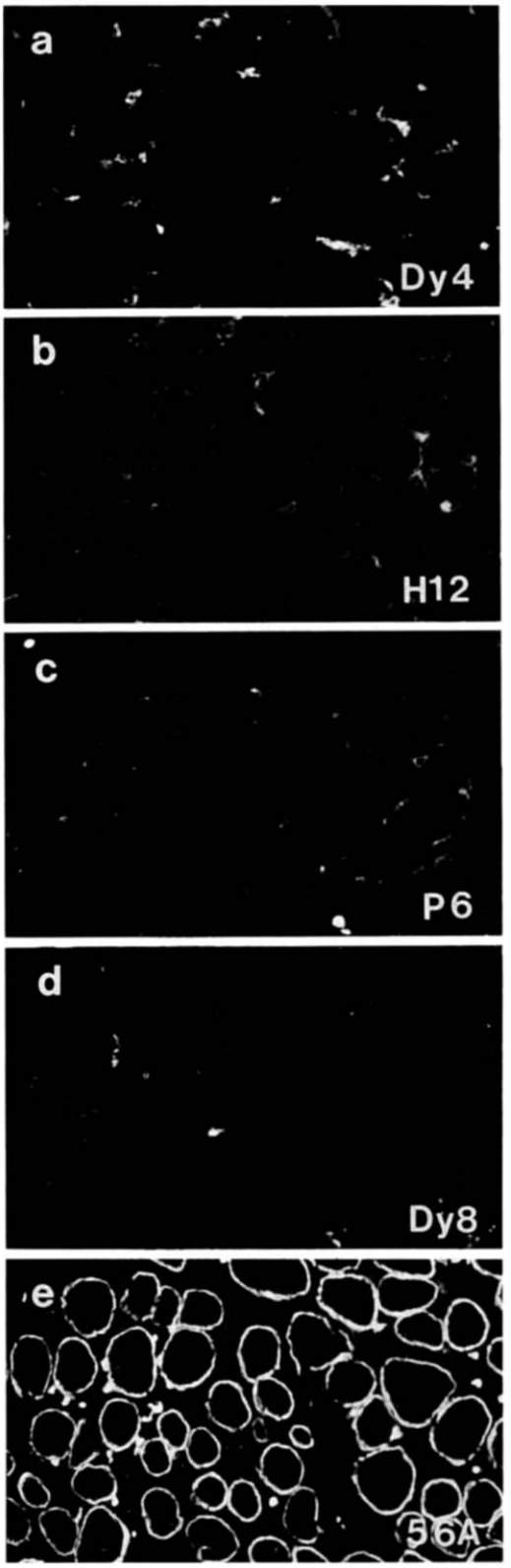

Fig. 10. Dystrophic dog biceps femoris 14 days post-injection of venom immunolabelled with antibodies to: (a)–(d) dystrophin Dy4, H12, P6, Dy8 respectively; (e) β-spectrin. Note the persistence of labelling with H12 and P6 on regenerating fibres but uniform labelling with β-spectrin. × 195.

Fig. 11. Dystrophic dog biceps femoris 28 days post-injection of snake venom immunolabelled with antibodies to dystrophin, Dy4, showing weak labelling of most regenerating fibres. × 195.

from the plasma membrane very early. Our results are similar to those reported by Vater *et al.* [22], who showed that dystrophin breaks down a few hours after injection of venom in rat muscle. As β-spectrin is lost at a similar rate to dystrophin, both proteins can be used as markers for the integrity of the plasma membrane and for assessing the preservation of tissues. Spectrin is a useful control for the immunostaining of dystrophin and its use avoids false negative results due to degradation or necrosis.

In summary, our results have shown that muscle from both normal and dystrophic dogs can regenerate rapidly and that the defect in dystrophin expression in dystrophic dogs does not impede regeneration. In normal dogs the appearance of dystrophin precedes that of β-spectrin and the normal immunostaining pattern of both is restored by 10–14 days. Weak immunolabelling with antibodies to β-spectrin is a feature of normal and dystrophic immature fibres. In dystrophic dogs regenerating fibres bind antibodies to dystrophin at early stages, which may relate to isoforms of dystrophin, but this binding is insufficient to influence the interpretation of myoblast transfer experiments.

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