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A homologue of dystrophin is expressed at the neuromuscular junctions of normal individuals and DMD patients, and of normal and *mdx* mice

Immunological evidence

F. Pons[1], N. Augier[2], J.O.C. Léger[2], A. Robert[2], F.M.S. Tomé[3], M. Fardeau[3], T. Voit[4], L.V.B. Nicholson[5], D. Mornet[2] and J.J. Léger[2]

[1] *Pathologie Générale, Faculté de Médecine, Place Henri IV, 34000 Montpellier, France,* [2] *Pathologie Moléculaire du Muscle, INSERM U300, Faculté de Pharmacie, Av. Ch. Flahaut, 34060 Montpellier cedex 1,* [3] *Développement, Pathologie, Régénération du Système Neuromusculaire, INSERM U153, 17 rue du Fer à Moulin, 75005 Paris, France,* [4] *Universitäts-Kinderklinik, Moorenstr. 5, D-4000 Düsseldorf, Germany,* and [5] *Muscular Dystrophy Group Research Laboratories, Regional Neurological Centre, New-Castle General Hospital, Newcastle upon Tyne NE4 6BE, UK*

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Polyclonal and monoclonal antibodies, which recognize different regions and epitopes of the dystrophin molecule, bind to a protein of M_r 400000 which is present in extracts of *mdx* muscle from regions which contain neuromuscular junctions (NMJ) and is absent from those which do not. This NMJ-associated homologue of dystrophin has at least 2 epitopes which are different to the usual Xp21 form of dystrophin expressed along the sarcolemma of muscle fibres in normal muscles. This protein is also expressed at the NMJ of a DMD patient who lacks the first 52 exons of the Xp21 dystrophin gene and it must therefore be translated from a different gene transcript.

Dystrophin; Neuromuscular junction (NMJ); Dystrophin related protein (DRP)

1. INTRODUCTION

Dystrophin is the protein product of a gene localized at the locus Xp21 [1,2] and expressed at the periphery of skeletal muscle fibers [3–7]. This gene is affected in Duchenne muscular dystrophy (DMD) patients and *mdx* mice through mutations which disrupt the reading frame of the transcript preventing dystrophin expression in the corresponding skeletal muscles [8–14]. High concentrations of dystrophin (or a dystrophin-like protein) have been observed at the neuromuscular junctions (NMJ) in normal rats, rabbits or mice as well as in the electric organ of *Torpedo marmorata* [15–18]. Fardeau et al. [19] recently reported a positive immunofluorescence response at the NMJ of DMD patients and *mdx* mice, using antibodies raised against the central and distal parts of chicken skeletal muscle dystrophin [20,21].

We now observe a protein of M_r 400000 which is present in extracts of *mdx* muscle from regions which contain NMJs and is absent from those which do not. This NMJ-associated homologue of dystrophin has at least 2 epitopes which are different to the usual Xp21 form of dystrophin expressed along the sarcolemma of muscle fibres in normal muscles. This protein is also expressed at the NMJ of a DMD patient who lacks the first 52 exons of the Xp21 dystrophin gene.

Correspondence address: F. Pons, INSERM U300, Faculté de Pharmacie, Av. Ch. Flahaut, 34060 Montpellier cedex 1, France. Fax: (33) 67042140

2. MATERIALS AND METHODS

2.1. *Biopsy materials and diagnoses*

The patient was a 9-year-old male who has followed a severe Duchenne course and is also mentally retarded. No hybridization signal was obtained with the DMD cDNA probes 1–2a, 2b–3, 4–5a, 5b–7 and 8 (American Type Culture Collection, depositor L.M. Kunkel) on Southern blotting. According to PCR, no signal was obtained for exons 3, 4, 6, 8, 12, 17, 19, 44, 45, 48, 50, 51, 52 (probes were gift of Drs J. Chamberlain [22] and A. Beggs). The first exon detected was exon 60. Consequently this DMD patient carries a deletion spanning at least the first 52 exons of the 75+ which form the Xp21 transcript [23]. The control muscle biopsy was from a patient with an ocular myopathy and no apparent dystrophin deficiency.

2.2. *Preparation of polyclonal and monoclonal antibodies*

Polyclonal antibodies were generated from rabbits immunized with 3 recombinant fusion proteins constructed from different fragments of chicken skeletal muscle dystrophin cDNA [20] and spanning the amino-terminal domain (serum A: residues 43–760), part of the central rod domain (serum C: residues 1173–1728) and the entire carboxy-terminal domain (serum H: residues 3357–3660) of chicken dystrophin [20,21] (N. Augier et al., unpublished). MAb Dy4/6D3 and MAb Dy8/6C5 were obtained by lymphocyte hybridation from mice immunized either by the 30 kDa fusion protein spanning a part of the central rod domain of mouse cardiac muscle dystrophin

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(residues 1181–1388) [2], and by a synthetic peptide consisting of the last 17 amino acids at the C-terminus of the human skeletal muscle dystrophin respectively [11,12]. MAb Dy18 was obtained by lymphocyte hybridation from a mouse immunised by the recombinant fusion protein containing the entire carboxy-terminal domain (serum H: residues 3357–3660) of chicken skeletal dystrophin [20].

2.3. *Immunofluorescence and Western blotting*

Indirect immunofluorescence was performed on 10 μm thick sections of mouse and human muscles frozen in isopentane, as in [24]. For Western blotting, 20 serial 10 μm thick cryostat sections, adjacent to sections used for control immunofluorescence studies (not shown), were homogenized in 40 μl of gel loading SDS-containing buffer according to the previously described Western blotting micro procedure [24]. One of 5 cryostat sections were immediately examined for their possible NMJ content by determining acetylcholine esterase activity [25]. Absence of dystrophin at the periphery of muscle fibres was verified in all *mdx* muscles used for homogenates. 10 μl samples were applied on 0.75 mm SDS-polyacrylamide gels, using a 2% to 7% gradient gel and non-stacking gel containing 25% glycerol. The amount of muscle extracts applied were a posteriori controlled by measuring their Coomassie detected myosin content. Transfer and immunostaining procedures were performed as in [24]. Alkaline phosphatase labelled anti-rabbit or anti-mouse antibodies (Miles) were used for labelling.

3. RESULTS AND DISCUSSION

We recently observed intense positive immunofluorescence reactions at the NMJ of skeletal muscles from DMD patients and *mdx* mice using 3 different polyclonal antisera raised against the N-terminal end (antiserum A), one part of the central rod-like domain (antiserum C) and the C-terminal end (antiserum H) of chicken skeletal muscle dystrophin [19]. These regions show 80%, 75% and 95% conservation respectively, with the human skeletal muscle sequence [20]. To determine the relative molecular mass of the protein(s) detected by indirect immunofluorescence with the 3 dystrophin antisera [19], extracts prepared from innervated or non-innervated regions of diaphragm muscles from normal and *mdx* mice were analyzed by Western blotting. A thin immunoreactive band of approximately M_r 400000 was detected in extracts of innervated *mdx* mouse muscle by the polyclonal antisera raised against the central (Fig. 1B, lane 1) and distal regions (not shown) of chicken skeletal muscle

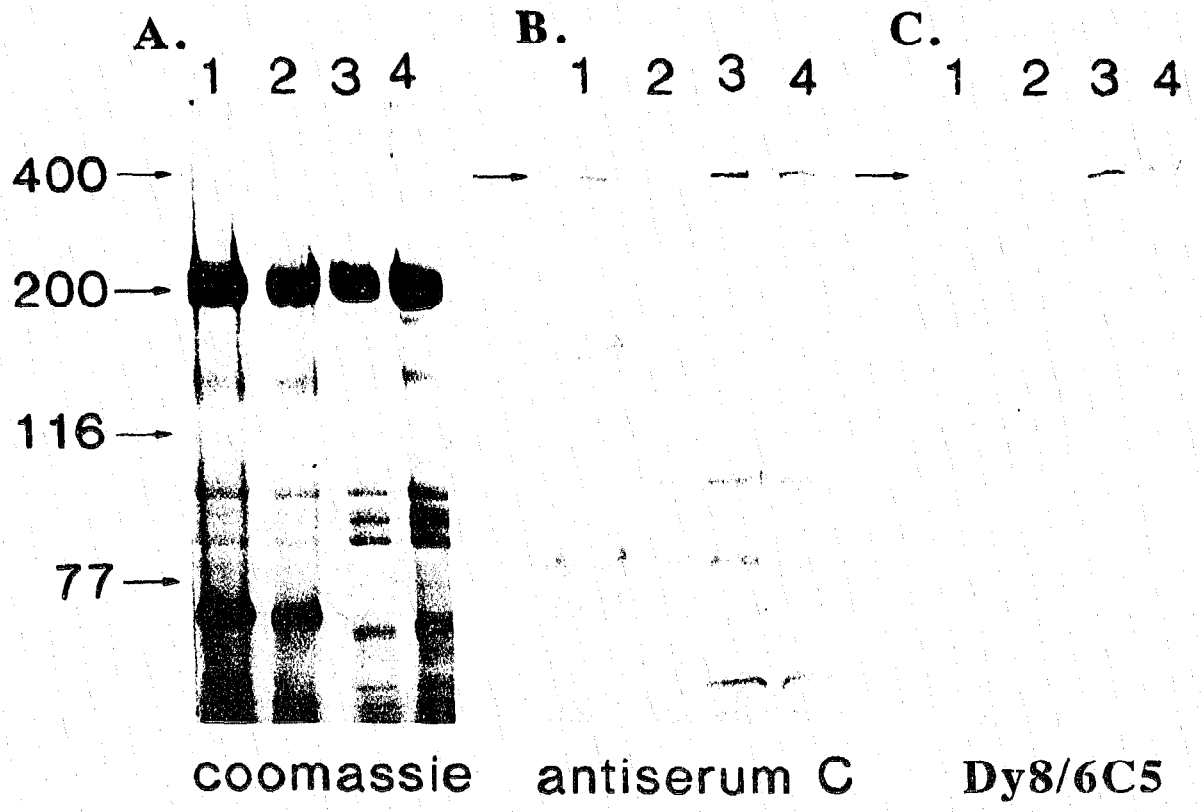

Fig. 1. Comparative immunoanalyses of dystrophin expression at the NMJ in the diaphragm muscle of *mdx* and control mice. Total extracts from cryostat sections of innervated and non-innervated muscle regions of an adult *mdx* mouse (lanes 1, 2 respectively) and of an adult C57BL/10 control mouse (lanes 3, 4, respectively) were compared by Western blotting analysis using either polyclonal antibodies raised against 3 different regions of the chicken skeletal muscle dystrophin [20,21] or 3 MAbs which recognise different domains of human skeletal muscle dystrophin [11,12]. The same amount of extracts were applied in all panels. Panel A shows the Coomassie blue staining of the muscle extracts; panel B shows the labelling observed with the polyclonal antiserum raised against the central part of the chicken dystrophin (serum C) [21]; and panel C shows the labelling observed with MAb Dy8/6C5 raised against the last 17 C-terminal residues of human skeletal muscle dystrophin.

dystrophin. The 400K band was absent in extracts made from non-innervated regions of *mdx* mouse muscle (Fig. 1B, lane 2). A thick immunoreactive band of M_r approximately 400000 was detected in extracts from innervated and non-innervated regions of the diaphragm muscle from normal mice, using antisera raised against the central (Fig. 1B, lane 3, 4) and distal (not shown) regions of chicken skeletal muscle dystrophin. The thin immunoreactive band detected in the extracts made from innervated regions of *mdx* mouse muscle migrated roughly to the same position as the major dystrophin band found along the sarcolemma of normal skeletal muscle fibres. It therefore has a similar if not identical relative molecular mass. Other bands with lower M_r were weakly stained in non-innervated or innervated muscle regions of normal and *mdx* muscle by the polyclonal antisera. These bands could be due to some dystrophin proteolysis or to some weak crossreactivities of the polyclonal antisera with other muscle proteins [5,6,26,27]. Nevertheless, the most intense immune signal detected in the extracts made from innervated regions of *mdx* mouse muscle was clearly found at the level of the 400K band. Considering the current evidence from blots and the previous immunofluorescence experiments [19], we suggest that the immunoreactivity detected by both methods indicates the existence of an NMJ associated protein which has structural homology to dystrophin in 3 different domains.

To test the immunological homology between the dystrophin expressed either at the subsarcolemmal level and/or in the NMJ of skeletal muscles, 3 monoclonal antibodies (MAbs) reacting with the conventional form of muscle dystrophin were applied on the same extracts made from innervated and non-innervated regions of *mdx* and normal mouse muscles. The MAbs were Dy4/6D3 [11] which recognises an epitope in the central rod domain (30K fusion protein [2]) in a region spanned by polyclonal antiserum C; Dy8/6C5 which recognises an epitope within the last 17 carboxy-terminal dystrophin residues in a region spanned by antiserum H; and Dy18, which recognises an epitope in the last 303 residues (3357–3660) of the carboxy domain of chicken skeletal muscle dystrophin. Unlike Dy8/6C5, Dy18 does not react in ELISA experiments with a synthetic peptide consisting of the last 17 amino acids of the C-terminus of the skeletal muscle dystrophin. MAb Dy18 reacted with the thin NMJ-associated 400K band previously observed in *mdx* muscle with the 3 polyclonal antisera (not shown, as in Fig. 1B). In contrast, neither of the 2 other MAbs reacted with the NMJ-associated 400K band in *mdx*

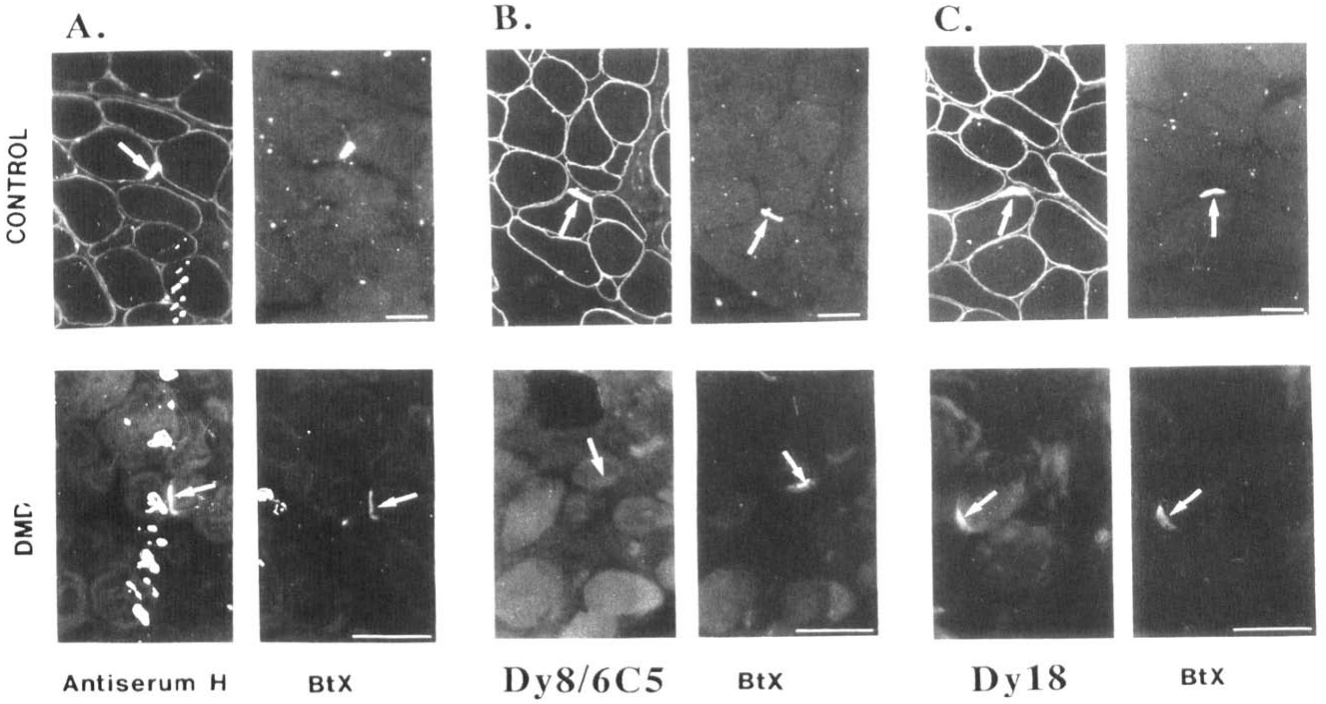

Fig. 2. Comparative immunocytochemical analysis of serial innervated muscle sections from a 9-year-old DMD patient with a deletion spanning at least the 52 first exons of the Xp21 gene and from a non dystrophin-deficient adult control. The sections were incubated with the polyclonal antibodies raised against 3 different regions of the chicken skeletal muscle dystrophin [19–22] or with 3 MAbs which recognise different domains of human skeletal muscle dystrophin [11,12]. Each panel presents the NMJ localization by rhodamine labelled a-bungarotoxin (BtX) [19]. Panel A shows the labelling observed with the polyclonal serum raised against the C-terminal part of the chicken dystrophin (serum H) [19–21]. Panel B shows the labelling observed with MAb Dy8/6C5 raised against the last 17 C-terminal residues of human skeletal muscle dystrophin [12]. Panel C shows the labelling observed with MAb Dy18 raised against the C-terminal domain (residues 3357–3660) of chicken dystrophin [20]. Bars = 10 μm.

muscle (Fig. 1C, lanes 1 and 2). All 3 MAbs reacted with the dystrophin expressed in normal mouse muscles (Fig. 1B and C, lanes 3 and 4). We conclude that the NMJ-associated homologue of dystrophin is immunologically (structurally) distinct from Xp21 encoded dystrophin in at least 2 epitopes from different domains and is similar in at least one epitope at the C-terminal end.

The immunoanalysis was confirmed by comparing the reactivity of the same set of 3 polyclonal antisera and 3 MAbs on cryostat sections of skeletal muscle from a non dystrophin-deficient control and from a DMD patient with a deletion spanning at least the first 52 exons of the 75+ which form the Xp21 transcript [23]. Positive immunoreactivity was observed at the NMJ of the DMD patient and at the NMJ and sarcolemma of normal muscle using the polyclonal antisera (see Fig. 2A, the reaction with the antiserum against the C-terminal end) and MAb Dy18 (Fig. 2C). In contrast, both MAb Dy4/6D3 and Dy8/6C5 stained the periphery of normal muscle fibres but not NMJ of the DMD patient. Staining with both MAbs was detected at the NMJ of the normal human muscle, indicating that normal Xp21 dystrophin is also expressed at the NMJ (see Fig. 2B, the reaction with the MAb raised against the last 17 C-terminal dystrophin residues).

We have thus been able to demonstrate the existence of an M_r 400000 NMJ-associated homologue of dystrophin which is expressed in muscle from DMD patients and *mdx* mice as well as from normal controls. The protein was detected by antisera which were generated by immunization with fusion proteins containing cDNA sequences from 3 distinct regions of chicken skeletal muscle dystrophin. The antisera may contain 2 populations of antibodies: those which demonstrate conventional labelling of dystrophin at the sarcolemma, and those which show labelling which is restricted to neuromuscular junctions. It is well known that such recombinant fusion proteins may generate antibodies to dystrophin plus other structurally related proteins [26,27].

The NMJ-associated protein we describe here could not be an isoform of Xp21 encoded dystrophin generated by alternative splicing because the NMJ protein was detected in muscle from a DMD patient in whom the Xp21 locus was substantially deleted. Such a mechanism could have been hypothesised for the NMJ protein detected in *mdx* mouse muscle, since a simple point mutation has been identified as the gene defect in this strain [28].

A second possibility is that this NMJ protein is the chromosome 6-encoded homologue with a C-terminal sequence similar to that of Xp21 encoded dystrophin which has been identified in fetal muscle [29]. Khurana et al. have recently isolated part of this chromosome 6 encoded cDNA by polymerase chain reaction cloning, expressed this as a recombinant bacterial protein and raised polyclonal antibodies which recognise a 'dystrophin related protein' (DRP) [30]. The 'DRP' is reported to exactly co-migrate with dystrophin as our NMJ-associated homologue. We have recently determined that MAb Dy18 (but neither of the other MAbs) reacts with the recombinant bacterial protein issued from the chromosome 6 encoded cDNA and with a protein of M_r similar to NMJ protein or DRP in brain and liver in normal mice (data not shown). Therefore the NMJ homologue appears to share at least one common epitope with the DRP and with the conventional dystrophin.

The third possibility is that the NMJ-associated protein we describe is a member of the family of dystrophin homologues which is encoded by a gene that has not yet been identified. It seems possible that the function of such an NMJ homologue might be related to the maintenance of membrane stability during ion exchange activities and denervation or cross-innervation experiments might be informative on this point. The generation of monoclonal antibodies which are specific for the chromosome 6 protein or the NMJ-associated protein, and which react on both blots and tissue sections, will be essential for us to distinguish between these homologues and to investigate the potential role of these proteins in normal and diseased tissues.

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