Dystrophin analysis using a panel of anti-dystrophin antibodies in Duchenne and Becker muscular dystrophy

Francesco Muntoni, Anna Mateddu, Carlo Cianchetti, Maria Giovanna Marrosu, Angela Clerk, Milena Cau, Rita Congiu, Antonio Cao, Maria Antonietta Melis

Abstract
Dystrophin, the protein product of the Duchenne muscular dystrophy (DMD) gene, was studied in 19 patients with Xp21 disorders and in 25 individuals with non-Xp21 muscular dystrophy. Antibodies raised to seven different regions spanning most of the protein were used for immunocytochemistry. In all patients specific dystrophin staining anomalies were detected and correlated with clinical severity and also gene deletion. In patients with Becker muscular dystrophy (BMD) the anomalies detected ranged from inter- and intra-fibre variation in labelling intensity with the same antibody or several antibodies to general reduction in staining and discontinuous staining. In vitro evidence of abnormal dystrophin breakdown was observed reanalysing the muscle of patients, with BMD and not that of non-Xp21 dystrophies, after it had been stored for several months. A number of patients with DMD showed some staining but this did not represent a diagnostic problem. Based on the data presented, it was concluded that immunocytochemistry is a powerful technique in the prognostic diagnosis of Xp21 muscular dystrophies.

The underlying biochemical defects responsible for Duchenne and Becker muscular dystrophy are abnormalities of dystrophin, the protein product of the Duchenne muscular dystrophy (DMD) gene. Immunocytochemical studies have shown that in normal muscle, dystrophin is visualised as a continuous ring of staining at the periphery of every muscle fibre; in DMD muscle the staining in most cases is totally absent, although the occurrence of scattered positive fibres has been reported. In the majority of patients with BMD a patchy or discontinuous staining pattern around most fibres has been described, the staining intensity being usually fainter than that seen in normal muscle; furthermore a variability in staining between individual fibres has been recorded, although no abnormalities were detected in several patients with mild BMD. In most of these studies only one N-terminal antibody was used.

In this study we evaluated a group of 19 patients with Xp21 muscular dystrophy, displaying a continuous spectrum of severity ranging from severe DMD to asymptomatic patients. We correlated the pattern of dystrophin immunofluorescence abnormalities, studied with antibodies directed towards seven different regions of dystrophin cDNA, with clinical severity as well as type and extension of gene deletions, when found. Our results suggest that the combined use of several anti-dystrophin antibodies is more informative than the use of one or a small number of antibodies for the prognostic diagnosis of Xp21 disorders.

Materials and methods
Each biopsy sample was tested with 7 different anti-dystrophin antibodies, two of which were monoclonal antisera (Dys 1 and Dys 2, Novocastra Laboratories, UK). The polyclonal antibodies 90K (1), H12 and P6 were the generous gift of P Strong and T Sherratt, London, while antibody P20 and D1-2 were donated by I Ginjaar, Leiden (The Netherlands) and A Mora and F Cornello, Milan (Italy), respectively. The mouse peptides correspond to amino acids 53–664 (D1–2), 407–815 (90K), 1181–1388 (Dys 1), 1750–2248 (P20), 2542–3025 (H12), 2814–3028 (P6), 3688–3685 (Dys 2). In fig 1 the region of the protein recognised by each antibody is indi-

ANTIDYSTROPHINANTIBODIES

Figure 1. The four domains of dystrophin with the region of the protein recognised by each of the seven antibodies used is reported.
Dystrophin abnormalities in BMD and intermediate patients

Table 1  Dystrophin abnormalities on tissue sections in BMD and intermediate patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Phenotype</th>
<th>Exon deleted</th>
<th>Reading-frame</th>
<th>Pattern of fibre labelling</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD 1</td>
<td>27</td>
<td>Moderate</td>
<td>48-50</td>
<td>-</td>
<td>80% continuous, of variable intensity; 10% discontinuous, moderate intensity.</td>
</tr>
<tr>
<td>BMD 2</td>
<td>8</td>
<td>Preclinical</td>
<td>48-51</td>
<td>+</td>
<td>100% normal for all antibodies except for H12 (most fibres showed variable intensity with this antibody).</td>
</tr>
<tr>
<td>BMD 17</td>
<td>10</td>
<td>Preclinical</td>
<td>non deletion</td>
<td></td>
<td>90% continuous, of variable intensity; 8% discontinuous, faint; 2% of negative fibres. (*)</td>
</tr>
<tr>
<td>BMD 19</td>
<td>12</td>
<td>Mild</td>
<td>48-51</td>
<td>+</td>
<td>75% continuous, of variable intensity; 23% discontinuous, of moderate or faint intensity; 2% of negative fibres. (*)</td>
</tr>
<tr>
<td>B/MDD 35</td>
<td>12</td>
<td>Intermediate</td>
<td>Non-deletion</td>
<td></td>
<td>5% continuous, variable intensity; 70% discontinuous, faint or very faint; 25% of negative fibres.</td>
</tr>
<tr>
<td>BMD 51</td>
<td>9</td>
<td>Mild</td>
<td>49-51</td>
<td>+</td>
<td>94% continuous, variable intensity; 6% discontinuous, moderate intensity. (*)</td>
</tr>
<tr>
<td>BMD 63</td>
<td>9</td>
<td>Intermediate</td>
<td>3-7</td>
<td>-</td>
<td>100% discontinuous, faint or very faint. No immunoreactivity was detected with D1-2 antibody.</td>
</tr>
<tr>
<td>BMD 73</td>
<td>57</td>
<td>Mild</td>
<td>non deletion</td>
<td></td>
<td>70% continuous, normal or variable intensity; 30% discontinuous, faint or very faint. (*)</td>
</tr>
<tr>
<td>B/MDD 87</td>
<td>11</td>
<td>Intermediate</td>
<td>3-9</td>
<td>+</td>
<td>100% discontinuous, faint or very faint. 99% of fibres showed no immunoreactivity with D1-2 antibody.</td>
</tr>
<tr>
<td>BMD 89</td>
<td>25</td>
<td>Mild</td>
<td>non deletion</td>
<td></td>
<td>98% discontinuous, moderate intensity; 2% of negative fibres.</td>
</tr>
<tr>
<td>BMD 92</td>
<td>7</td>
<td>Preclinical</td>
<td>Non deletion</td>
<td></td>
<td>88% continuous, variable intensity; 8% discontinuous, moderate; 4% negative fibres.</td>
</tr>
</tbody>
</table>

The symbol + or − under the heading READING-FRAME indicates the maintenance of reading frame.

The asterisk (*) indicates that reduction in immunostaining was detected using C-terminal antibodies or antibodies distal to a deleted portion of the protein.

Results

Pattern of dystrophin abnormalities in BMD patients

Various abnormalities were detected in the muscle of patients with BMD (table 1), the most frequent being an immunostaining variability between individual fibres. In contrast to what is consistently found in normal muscle (that is, identical levels of immunostaining in all fibres, fig 2), the inter fibre variability in staining, using any antibody, was the most frequent abnormality encountered in the vast majority of BMD patients (table 1, fig 3). A discordance in staining between the various antibodies was the second most frequent abnormality. This was also noted in patients with very mild or preclinical phenotypes, in which the use of just one or two antidystrophin antibodies gave normal results when taken individually. Only the comparison of results obtained with antibodies raised against different portions of the protein was revealing in these mildly affected individuals. Several patients had deletions of the cDNA in a region partially encompassed by one of the antibodies used (for example BMD 2 for H12 antibody) which gave a very faint signal in this subset of cases. A discontinuous labelling of the membrane was the third most common abnormality, but was confined to patients in the moderate/severe range of BMD (fig 4); rare dystrophin negative fibres were detected in these subjects (table 1). No specific immunofluorescence abnormality was detected in the 25 patients with non-Xp21 dystrophies except...
for the absence of dystrophin in fibres in an advanced state of necrosis. The same fibres lacked surface immunolabelling with $\beta$-spectrin, indicating a complete loss of their plasma membrane. Western blot analysis performed with 90K and P6 antibodies on all our patients with BMD confirmed the dystrophin abnormalities detected with immunofluorescence (data not shown).

**In vitro evidence of abnormal dystrophin breakdown in BMD muscle**

When the muscle samples of patients with BMD (when stored at $-30^\circ$C) were retested for dystrophin immunoreactivity, a progressive fading of signal intensity was detected after two to 12 months. The degree of immunoreactivity loss was maximal when using antibodies partially encompassed by or distal to a deletion; some variation was also noted using C-terminal antibodies. The same phenomenon (that is, greater loss of immunoreactivity using C-terminal as opposed to N-terminal antibodies) was also noted in patients with BMD in whom no deletion was found. The decrease in immunoreactivity was maximal when the muscle was stored already sectioned; control muscle, or muscle belonging to a patient with non-Xp21 muscle disorders, stored identically, showed no evidence of loss of signal after the same time lapse or even longer (up to a maximum of 2-5 years). No decrease in immunoreactivity with time was noted in serial sections using antibodies to $\beta$-spectrin.

**Pattern of dystrophin abnormalities in DMD patients**

The greater number of boys with DMD had no detectable dystrophin with most of the antibodies used (table 2). In a few patients (3/10) a very faint immunoreactivity, correctly located at the periphery of the muscle fibres, was detected in the majority of fibres using D1–2 and 90K N-terminal antibodies, but not with all remaining antibodies. The same phenomenon was observed in the muscle of a DMD foetus. A progressive loss of this weak immunoreactivity was also observed in this subgroup of patients with DMD when retested after 3 weeks and up to a couple of months.

When muscle of boys with DMD was analysed using the complete panel of anti-dystrophin antibodies, the following exceptions to the rule “dystrophin absence = DMD” were detected:

a) Scattered positive fibres in DMD. Occurrence of scattered positive fibres (3/10) was noted in the majority of cases; sometimes positivity was followed for more than 200µm before the positive signal was lost. The same phenomenon was found in a DMD foetus (14 weeks), in which the presence of rare positive myotubes (as opposed to negative myotubes) was detected.

b) Dystrophin immunoreactivity in the muscle spindle of a boy with DMD. The intrafusal fibres of a muscle spindle of a boy with DMD (patient 41) were very brightly stained with all
antibodies used. In figure 6 immunoreactivity obtained with 90K antibody is shown: perinuclear staining was detected only with this antibody. The strong positive immunoreactivity was followed, in serial sections, for approximately 140μ (20 slices), but disappeared thereafter in all fibres.

c) Split fibres. In DMD, the newly synthesised membrane of a splitting fibre frequently displayed low levels of dystrophin immunoreactivity although the periphery of muscle fibres was negative (fig 7). This positivity was detected with all antibodies. Splits (and vacuoles) were always strongly positive in BMD and non-Xp21 disorders.

Very weak immunostaining was detected in the three patients with an intermediate phenotype. Patient 86 (deleted for exons 3–9) and 63 (exons 3–7 deleted) showed no immunoreactivity with the most N-terminal antibody D1–2, raised against an epitope partially deleted in these two patients (fig 8a), but starting from antibody 90K and as far as the most C-terminal antibody a clear, although very weak immunoreactivity was detected (fig 8b); several dystrophin-negative fibres were also present (table 1). Patient 35, who had no deletion of the cDNA showed very weak immunostaining with all antibodies.

Fifty per cent of patients with DMD and BMD exhibited a deletion. In tables 1 and 2 the number of exons deleted is recorded. The deletion altered the reading frame in all patients with DMD but in none of the BMD patients we analysed with the exception of patient 1, a 27 years old ambulant patient with Becker muscular dystrophy: he was missing exons 48–50, causing a frame-shifting. Of the two subjects with an intermediate phenotype with a deletion, one had an in-frame deletion (subject 87, exons deleted 3–9), while the other carried an out-of-frame deletion (case 63, exons deleted 3–7) (table 2).

Discussion

Dystrophin deficiency has been well documented as the underlying cause of Duchenne muscular dystrophy by both immunofluorescence and immunoblot analysis.3-14 Abnormalities in the quantity and/or quality of dystrophin have also been well characterised in patients with BMD.3-6 In the main part of the original studies only one (N-terminal) antibody was used. In this study we have selected a panel of 7 different anti dystrophin antibodies and immunofluorescence analysis in a group of 19 patients with Xp21 disorders.

In patients with BMD we were able to identify different kinds of abnormalities related to phenotype severity. We established that the most frequent abnormalities in our BMD patients were staining variability between individual fibres (using one antibody) and differences in staining intensity when several antibodies were employed. The use of a complete set of antibodies enabled us to identify very subtle anomalies that cannot be detected with the use of only one antibody in the BMD patients (our data and 8).

A further useful test to reveal abnormalities in patients with very mild BMD was that of restaining serial sections of muscle after storing

---

**Table 2** Dystrophin abnormalities on tissue sections in DMD patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Exon deleted</th>
<th>Reading-frame</th>
<th>Pattern of fibre labelling</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMD 8</td>
<td>4</td>
<td>35–44</td>
<td>–</td>
<td>1% continuous, variable intensity; 99% negative fibres.</td>
</tr>
<tr>
<td>DMD 9</td>
<td>9</td>
<td>44</td>
<td>–</td>
<td>2% continuous, variable intensity; 98% negative fibres.</td>
</tr>
<tr>
<td>DMD 41</td>
<td>6</td>
<td>non deletion</td>
<td>–</td>
<td>0-5% continuous, normal intensity (muscle spindles); 6% discontinuous, very faint (§); 93-5% negative fibres.</td>
</tr>
<tr>
<td>DMD 49</td>
<td>11</td>
<td>non deletion</td>
<td>–</td>
<td>2% discontinuous, very faint (§); 98% negative fibres.</td>
</tr>
<tr>
<td>DMD 60</td>
<td>7</td>
<td>non deletion</td>
<td>45–47</td>
<td>4% continuous, normal intensity; 4% discontinuous, moderate (§); 92% negative fibres.</td>
</tr>
<tr>
<td>DMD 62</td>
<td>13</td>
<td>45–47</td>
<td>–</td>
<td>1% discontinuous, very faint (§); 99% negative fibres.</td>
</tr>
<tr>
<td>DMD 84</td>
<td>7</td>
<td>non deletion</td>
<td>–</td>
<td>16% continuous, variable intensity (§); 1% normal intensity; 4% discontinuous, moderate (§); 79% negative myotubes.</td>
</tr>
<tr>
<td>FOETUS</td>
<td>14 weeks</td>
<td>48–58</td>
<td>–</td>
<td>Very weak immunoreactivity although the periphery of muscle fibres was negative (fig 7). This positivity was detected with all antibodies. Splits (and vacuoles) were always strongly positive in BMD and non-Xp21 disorders.</td>
</tr>
</tbody>
</table>

The symbol + or – under the heading READING-FRAME indicates the maintenance of reading frame. The symbol (§) indicates the some labelling was detectable only with N-terminal antibodies.

---

Figure 6 DMD muscle, 90K antibodies (320x). All intrafusal muscle fibres show a distinct immunoreactivity for the antibody, while extrafusal fibres are completely negative. The nuclear membrane of intrafusal fibres is also stained with this antibody.

Figure 7 DMD muscle, DYS 1 antibody (320x). Membrane of several splitting fibres is weakly stained. A necrotic fibres shows some non specific cytoplasmic staining.
minimum of two antibodies (one N-terminal and one C-terminal) should be obtained before a diagnosis of DMD is confirmed. In 20% of DMD cases that were analysed, a very weak immunostaining was observed only with antibodies raised against the N-terminal region. This is not surprising as we (and other authors) have demonstrated that low levels of dystrophin mRNA are produced in the majority of patients with DMD. We propose that the mRNA produced is effectively translated into a protein up to the mutation/translocation region; the truncated protein is rapidly degraded, but in rare instances is still detectable with antibodies raised to the protein portion proximal to the mutation (our data 6-8 19); interestingly, this truncated protein, missing the C-terminal domain involved in linking dystrophin to a sarcolemmal glycoprotein 20 is correctly localised at the periphery of the membrane. The occurrence of scattered, positive fibres in the muscle of patients with DMD is a well-known phenomenon; we show that positivity is detectable with all antibodies used, indicating that a crossreactivity with the recently described dystrophin-related protein 61 22 is highly unlikely. The presence of scattered positive myotubes was also observed in a DMD foetus; the occurrence of this phenomenon in a myotube may explain the finding of groups of positive fibres later on in development. We also provide the first evidence for dystrophin positive fibres of a muscle spindle in a boy with DMD. No such positivity was found in our other patients with DMD, or in another patient described by Tanaka 23. Positivity was detectable in all fusal fibres and with all antibodies for a distance of 140 µm, but disappeared thereafter in all fibres. Again, the fact that this positivity was detected with antibodies directed towards different dystrophin domains and that it disappeared after many serial sections, demonstrates that positivity was unlikely to be due to crossreactivity with a dystrophin-related protein. The newly synthesised membrane of splitting fibres was also found to be positive (with all antibodies) in several boys with DMD, who did not produce dystrophin in the periphery of the same fibres.

A close correlation was found between quantity of dystrophin immunoreactivity and phenotype severity: mild BMD patients produced more dystrophin than severe BMD, and the latter presented more detectable dystrophin than intermediate patients; there were a few boys with DMD in whom some immunoreactivity was observed with N-terminal antibodies and they had less dystrophin than intermediate individuals.

Of the individuals with deletions, two represented exceptions to the reading frame theory: one intermediate and one moderate BMD patient had frame-shift deletions instead of a translational in-frame mutation. In both of them a clear immunostaining was detected in all fibres with antibodies recognising portions of the protein distal to the deletion. One of the two patients (B/DMD 63), carried a deletion of exons 3–7; more than twenty individuals...
Dystrophin abnormalities in Xp21 muscular dystrophies

with the same deletion and an attenuated phenotype have been described. The finding that three minor additional in-frame transcripts were detectable in the muscle of these subjects may explain their relatively mild phenotype. The second patient, a 27 year old ambulant BMD, had an out of frame deletion involving exons 48–50. This mutation was associated with a severe DMD phenotype in recent study. We do not know the reasons for this discrepancy: identical deletions producing a DMD and a BMD phenotype have already been reported. In some instances, however, it is not possible to come to a conclusion about the reading frame as exons might be only partially deleted.

We conclude that the simultaneous use of several anti dystrophin antibodies is a powerful technique for detecting dystrophin abnormalities, including minor ones present in the muscle of patients with very mild phenotypes. Western blotting of muscle is useful especially when minor or no abnormalities can be detected with immunocytochemistry. However, the use of several antibodies greatly increases the likelihood of detecting some abnormalities with immunocytochemistry.

We thank the colleagues from the Paediatric and Paediatric Neurology Clinics of Sardinia for referring their patients (in particular we thank Professor S De Virgiliis, Professor C Mastroppolo and Dr V Serra). The financial support of Telethon-Italy to the project "Use of different strategies for the diagnosis of Duchenne and Becker carrier status" and of Regione Autonoma della Sardegna (R. 1980) is gratefully acknowledged.

Dystrophin analysis using a panel of anti-dystrophin antibodies in Duchenne and Becker muscular dystrophy.
F Muntoni, A Mateddu, C Cianchetti, M G Marrosu, A Clerk, M Cau, R Congiu, A Cao and M A Melis

*J Neurol Neurosurg Psychiatry* 1993 56: 26-31
doi: 10.1136/jnnp.56.1.26

Updated information and services can be found at:
http://jnnp.bmj.com/content/56/1/26

**Email alerting service**

Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

**Notes**

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/