Original articles

Immunohistochemical studies show truncated dystrophins in the myotubes of three fetuses at risk for Duchenne muscular dystrophy

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Abstract
We have performed immunohistochemical studies on muscle tissue of three 12 week old fetuses at risk for DMD, using antisera directed against regions located NH2-proximally and centrally in the rod shaped spectrin-like domain and against the COOH-terminus of dystrophin. All three fetuses had a family history of DMD. Truncated dystrophins were identified in all three cases by a positive reaction with the NH2-proximal antibody, different reactions with the central antibody, and a negative reaction with the COOH-terminal antibody. These data indicate that a panel of antibodies would, in principle, permit 'immunological' mapping of dystrophin mutations. This is diagnostically important in the 35% of families where no mutation is detectable at the DNA level. Secondly, by using this mapping technique it may also become possible to identify the at risk haplotype when DNA analysis is not informative. This may be of great value in DMD carrier detection.

X linked Duchenne muscular dystrophy (DMD) is the most common of all severe muscular dystrophies (1 in 3500 live male births) progressing to death in early adulthood. The milder Becker muscular dystrophy (BMD) has a later age of onset, but is caused by mutations in the same gene. DNA technology has allowed reliable diagnosis with more than 99% certainty in most familial DMD cases.1-3 Dystrophin,4 the DMD gene product, has been localised in the sarcolemma of the muscle cell.5-10 In muscle biopsies of Duchenne patients no dystrophin can be detected, whereas in BMD patients dystrophin of abnormal size or quantity is observed.7 8 11 The combined results of protein and DNA studies4 12-15 have confirmed the hypothesis16 that the Duchenne phenotype might be caused by an 'out of frame' mutation of the gene, although some exceptions have been found. The mutations produce a prematurely terminated protein, which is apparently unstable and thus degraded. Similarly, BMD is caused by 'in frame' mutations, resulting in a partly functional protein of altered molecular weight. In a preliminary study of muscle tissue of a fetus at risk for DMD, truncated dystrophin was detected immunohistochemically.17 The presence of abnormal dystrophin early in development indicates that its degradation takes place at a later stage. We describe here the presence of truncated dystrophins in two more fetuses at risk for DMD using antisera raised against three different regions of dystrophin and discuss the implications for fetal dystrophin analysis and the pinpointing of undetectable DMD mutations.

Materials and methods
Prenatal Diagnosis
Case 1 (fig 1A)
The proband (5) is a carrier and two of her brothers died of DMD at the ages of 14 and 19 years. A brother of the grandmother (carrier) also died very young.
The proband decided to terminate the male pregnancy after haplotyping had indicated a >99% risk of an affected fetus. DNA analysis of this mother indicated a duplication in the central part of the gene.\textsuperscript{14 17}

Case 2 (fig 1B)

The proband (12) had two brothers affected with DMD. The proband had a >99% carrier risk. No DNA abnormality could be detected in this family. After prenatal diagnosis had indicated the presence of a male fetus carrying the at risk haplotype, the parents decided to terminate the pregnancy.

Case 3 (fig 1C)

Three brothers of the proband (10) died of DMD. No mutation could be found in the DMD gene. Moreover, as rarely occurs, none of the available DNA markers was informative in the carrier grandmother (1), so the proband’s carrier risk remained unaltered at 50%. The only informative marker in the proband herself was the distal marker 782 (DXS885),\textsuperscript{7} located about 20 cM distally. Therefore, the reliability of the DNA test, to discriminate between a grandpaternal and grandmaternal X chromosome, would not exceed 80%. Since the grandmaternal 50% at risk X chromosome was detected in the male fetus (12), the prospective parents elected to terminate the pregnancy.

**DNA ANALYSIS**

DNA was isolated from whole blood or chorionic villus samples; restriction enzyme digestions and blot hybridisations were as described by Bakker et al.\textsuperscript{2} Restriction endonucleases were purchased from Pharmacia.

**IMMUNOHISTOCHEMICAL ANALYSIS**

The at risk fetuses were aborted by aspiration in the 12th week of pregnancy. Normal fetuses were obtained similarly after legal abortion for non-pathological reasons. Limited tissue material was available for the at risk fetuses. Intact leg segments of fetuses 2 and 3 and the controls were fixed for one to three days at room temperature in a mixture of methanol, acetone,

![Figure 1](https://example.com/figure1.png)

(A) Pedigree of family 1 segregating DMD. For carrier detection in this family the flanking probes pD2\textsuperscript{18} (DXS164) (E) and 754\textsuperscript{19} (DXS84) (C), the intragenic genomic probes p87\textsuperscript{20} 15 (L) and p87\textsuperscript{20} 1P (DXS164), and 5b-7cDNA probe\textsuperscript{2} (Z) were used. Daughters 5 and 6 have inherited the at risk chromosomes e-e-L-p-c. Prenatal diagnosis (7) was performed according to Bakker et al\textsuperscript{2} by using the flanking markers pD2 and 754. (B) Pedigree of DMD family 2. For carrier detection and prenatal diagnosis the markers pD2 (E), p87\textsuperscript{20} 15 (L), and 754\textsuperscript{21} 11 (T)\textsuperscript{20} were used. Daughter 12 and fetus 17 have inherited the at risk chromosome e-L-t. (C) Pedigree of family 3. For carrier detection and prenatal diagnosis the only informative marker was 782 (D). The fetus (12) has inherited one of the grandmaternal X chromosomes; the chance of this fetus being affected with DMD is 50%. To discriminate between the two maternal haplotypes genomic probes p87\textsuperscript{20} 15 (L) and 754 (T) are indicated in the pedigree.
acetic acid, and water (35:35:5:25 by vol) followed by dehydration in acidified 2:2-dimethoxypropane (Merck) and embedding in paraplast\(^{22}\) (Monoject). Because the DMD fetus of family 1 arrived frozen in liquid nitrogen, the tissue was freeze substituted for seven days at \(-40^\circ\text{C}\), in the same methanol/acetone/acetic acid/water mixture, dehydrated, and embedded in paraplast. For immunohistochemical staining, 7 \(\mu\)m thick sections of the legs of normal and DMD fetuses were deparaffinized and immunostained as described by Moorman et al.\(^{23}\) All the sera were diluted in PBS. The polyclonal antibodies used for incubations were affinity purified rabbit polyclonal antibodies, directed, respectively, against a 30 kD subpeptide consisting of amino acid 1181-1388\(^{24}\) (30 kD AB, fusion protein obtained from E Hoffman and antibody raised by KHF), a 59 kD subpeptide made of cDNA\(^2\) 5'-5 to 7'-0 kb, corresponding to amino acids 1750-2248, named P20-AB after the origin from the P20 deletion prone region,\(^{25}\) and against a peptide containing the last 17 amino acids, 3669-3685, of dystrophin (1460 AB, raised by EEZ-G).

**Results**

In the carrier female of family 1 an intragenic duplication was detected\(^{14}\) \(^{17}\) (fig 2). Families 2 and 3, however, belong to the 35% of families without detectable mutations in the DMD gene. Given the severity of the disease in all three families, the mutation in the gene probably results in a frameshift producing a truncated dystrophin. We performed immunohistochemical studies on muscle tissue of 12 week old fetuses, using three different polyclonal antibodies, directed against a NH\(_2\)-proximal segment (30 kD AB), a central segment (P20 AB), and the COOH-terminus (1460 AB) respectively (see Materials and methods). Fig 3 shows a control experiment to show the specificity of the central P20 antibody, with normal, mature muscle staining positively (A), with that of a female, manifesting DMD carrier (B) staining in a clear mosaic fashion,\(^{27}\) and with that of a DMD patient (C) showing absence of signal. Our parallel studies have shown that at 12 weeks of development dystrophin is not yet fully membrane associated, but is just beginning to appear.

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![Figure 2](http://jmg.bmj.com/) *A SfiI physical map in the DMD region.*\(^{26}\) Fully and partially digestible SfiI sites are represented by closed and open boxes, respectively. The SfiI fragment lengths are written in kb above the map, as are the locations of the genomic probes. Above the figure 65 genomic HindIII fragments are drawn, most of which correspond to only one exon. The DNA duplication in family 1 is situated in DE.

![Figure 3](http://jmg.bmj.com/) *(A) Normal muscle tissue (m quadriceps) incubated with P20, (B) muscle tissue from a female DMD carrier, and (C) muscle tissue from a DMD patient incubated with P20 antiserum.*
Figure 4  Dystrophin expression in a normal human fetus (many independent fetuses were tested with similar results) and three DMD fetuses. All fetuses were at about 12 weeks of development. Sagittal sections of fetal muscle leg tissue incubated with (A,D,G,J) affinity purified 30 kD AB, (B,E,H,K) P20 AB, and (C,F,I,L) affinity purified 1460 AB. The dystrophin domains, shown in the lower panel are as reported by Koenig et al. 26 Arrows indicate dystrophin. (Bar in L = 60 μm as in all panels.)
within the sarcoplasm between the distal nuclei and the myotendinous junction. This distribution was found in the present study for all three antibodies (fig 4 A, B, C, arrows). The proximal 30 kd antibody (AB) clearly stains the same region of the myotubes in all three Duchenne fetuses (fig 4 D, G, J). In contrast, with the P20 antibody, the dystrophin signal was absent from the myotubes of fetus 1 (fig 4 E), while it was present in fetuses 2 and 3 (fig 4 H, K). Finally, none of the three Duchenne fetuses showed any signal with the COOH-terminal antibody 1460 (fig 4 F, I, L). We noted that the muscle tissue of fetus 1 consistently and reproducibly stained very slightly above background with the P20 antibody, although this is difficult to visualise photographically (fig 4 E). As the P20 antibody spans the region of the gene duplication of this fetus, a small portion of the epitopes should still be detectable. More precise mapping of this duplication mutation has not yet been possible.

Discussion

By using three different antisera in our immuno-histochemical studies of DMD fetal tissue we have shown truncated dystrophins, lacking the COOH domain supposed to be involved in anchoring the protein with the plasma membrane via glycoproteins. Biochemical studies of Patel et al have also shown traces of dystrophin in some DMD fetuses. Our findings are consistent with the frame-shift hypothesis and can be reconciled with the absence of dystrophin in most older DMD patients by concluding that these truncated, unintegrated dystrophins are degraded at a later stage in muscle development. These results independently corroborate a biochemical study, in which up to 50% of the younger patients showed truncated dystrophins in their muscle tissue. In principle, some DMD cases could therefore be considered as integration defects rather than synthesis defects. We should point out that, although some dystrophin antibodies cross react with other proteins present in DMD patients, the antibodies used in the present study show no cross reaction in older DMD patients (30 kd, 1460, and this study for P20 (fig 3) and 1460 (fig 4 F, I, L)), nor in mdx mice. However, we cannot exclude the sharing of antigenic determinants between putative, exclusively embryonic, dystrophin-like proteins with a similar appearance early in embryogenesis. To investigate this further, additional biochemical studies of early fetal muscle would be required. This work has so far been hampered by the considerable dilution of the epitopes at this stage. The finding of different, truncated dystrophins obviously has great diagnostic significance for the 35% of DMD mutations not detectable by DNA analysis.

The application of these three antibodies allowed us to confine two hitherto undetectable mutations to the distal half of the protein. In the near future, a series of antibodies covering the entire dystrophin could be used to map mutations, which are otherwise undetectable, in a similar way. As well as indicating the position of mutations, this analysis also highlights the at risk haplotype. In family 3 (fig 1) the fetus has apparently inherited the affected haplotype. This raises the carrier risk of the mother to 100% and directly identifies the at risk haplotype. In a similar way to a biochemical study of Bieber et al carried out at 21 weeks of gestation, dystrophin analysis has settled the issue of carriership of the mother. This raises the question of the confidence with which the other haplotype can now be diagnosed as unaffected in subsequent pregnancies. A retrospective study of muscle material of aborted fetuses at 50% risk should settle this issue. Hopefully, a thorough regional epitope analysis in DMD/BMD fetuses and BMD patients will lead to a better understanding of the determinants of dystrophin stability. This seems both a rational and an obvious step on the way to therapeutic intervention in the course of this disease.

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