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Exertional rhabdomyolysis and exercise intolerance revealing dystrophinopathies

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Abstract Exercise intolerance associated with myalgias, muscle cramps or myoglobinuria may be associated with a dystrophinopathy. A search for abnormal dystrophin expression (using immunohistochemistry, immunoblot and DNA analysis) was carried out in a series of 15 patients. They were selected because they presented exercise intolerance, negative biochemical tests (lipid, glycogen and mitochondrial metabolism) and abnormal immunohistochemistry with at least one anti-dystrophin antibody (anti-Dys 1, rod domain; anti-Dys 2, C terminus; anti-Dys 3, N terminus). Lack of anti-Dys 1 immunoreactivity was seen in three patients and abnormal immunoreactivity with all three anti-dystrophin antibodies in two. Immunoblot confirmed the dystrophinopathy in these five patients only, and multiplex polymerase chain reaction DNA analysis revealed a deletion in the dystrophin gene in two of these patients, affecting the proximal part of the rod domain in one and the distal part of this domain in the other. The clinical, biological and histopathological features of the five patients reported here, together with the previous cases reported in the literature, are described and reveal that exercise intolerance associated with dystrophinopathy displays characteristic clinical, biological and immunohistochemical features and defines a new dystrophinopathy phenotype. The absence of staining in the rod domain provides a secure diagnosis of this syndrome. Dystrophinopathy is one etiology of idiopathic myoglobinuria, requiring genetic counseling.

Key words Dystrophin · Exercise intolerance · Immunohistochemistry

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Introduction

Exertional rhabdomyolysis and exercise intolerance such as post-exercise cramps and myalgias or post-exercise muscle stiffness are usually due to muscle metabolic dysfunction. However, extensive studies of the dystrophin gene have shown that besides the two major dystrophinopathy phenotypes represented by Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD), other phenotypes could occur (for reviews see [1, 6, 10]). They include exercise intolerance associated with myalgias, muscle cramps or myoglobinuria [9, 12, 13, 17, 19, 21]. Following this line of research, we have screened immunohistochemically for abnormal dystrophin expression in patients presenting exercise intolerance and normal muscle metabolism, as assessed by measurement of carnitine palmitoyltransferase (CPT), glycolytic enzymes and mitochondrial respiratory chain enzymes in their muscles. Specific monoclonal antibodies directed against the different parts of the dystrophin protein (the C and N termini and rod domain) were used. Fifteen patients were selected. Clinically, they all presented exercise intolerance, normal muscle metabolism and abnormal anti-dystrophin immunoreactivity with at least one anti-dystrophin antibody. Western blotting analysis and multiplex polymerase chain reaction (PCR) [3, 7] were conducted in all cases.

Material and methods

Patients and muscle biopsies

The patients (all males except cases 4 and 5) all presented exercise intolerance. Neurological examination, electromyographic studies and measurement of serum creatine kinase (CK) were performed in all patients for at least 3 months after the exercise intolerance episode.

Muscle biopsy samples were taken under local anesthesia from the following muscles: quadriceps for patients 1–3, 6–8, 12 and 13, deltoid for patient 4, biceps brachialis for patients 5, 11 and 14, gastrocnemius for patients 9 and 10 and in peroneus longus for patient 15. Samples were frozen in isopentane cooled by liquid nitrogen and stored at –80°C. Routine diagnostic histoenzymology was carried out on all specimens.

Muscle metabolism was normal in all cases. It was assessed by measurement of CPT [22], glycolytic enzymes [14] and mitochondrial respiratory chain in the muscles [5]. Specific measurement of CPT was performed because CPT deficiency is one of the major causes of myoglobinuria [23].

Antibodies

Monoclonal antibodies directed against different regions of the dystrophin were purchased from Novocastra Laboratories. Anti-Dys 1 antibody reacts with the rod domain. Anti-Dys 2 antibody reacts with the C-terminal and anti-Dys 3 antibody with the N-terminal region of the protein. These antibodies were used at 1:2 dilution for immunohistochemistry and at 1:10 for immunoblot analysis. In addition, anti-spectrin (NCL SPEC 1) and anti-utrophin (NCL DRP 2) were purchased from Novocastra Laboratories and used at 1:50 dilution.

Immunohistochemical procedure

Serial cryostat sections ($n = 7$; 6 μm thick) were prepared from all muscle specimens. The serial sections were stained with hematoxylin-eosin (H&E) and reacted with anti-Dys 1, anti-Dys 2, anti-Dys 3, anti-spectrin antibodies and controls. In addition, anti-utrophin immunoreactivity was performed for some of muscle specimens (cases 1, 8, 11, 12, 15), but not for the other cases for technical reasons. Immunoperoxidase staining was performed using the streptavidin-biotin-peroxidase complex (Dako-LSAB KO 680 Kit) method. Peroxidase staining was localized using 3-amino-ethylcarbazole (AEC) as substrate. Controls included omission of primary antibodies and irrelevant IgG. Immunohistochemistry was also performed on two normal muscle biopsy samples and two DMD muscle biopsy specimens taken as positive and negative controls, respectively.

Muscle extracts and immunoblot detection

Immunoblot analysis was performed for all the 15 selected muscle specimens and for one normal control. Muscle extracts were prepared according to the technique previously described by Dechesne et al. [8]: 10 μl of each sample was applied, in triplicate, onto 6.65% resolving SDS-polyacrylamide gels. Separated proteins were electroblotted onto nitrocellulose sheets (Amersham, Chicago, Ill.). Ponceau red staining of myosin heavy chain served as control for the muscle protein content of each lane. The sheets were then processed according to the previously described method [11]. They were probed with anti-Dys 1, -Dys 2 or -Dys 3 antibodies.

DNA analysis

PCR analyses were performed on DNA extracted from the muscle biopsy samples using 18 primers from exons 4, 8+9, 12, 17, 19, 44 and 51 [7] as well as exons 3, 6, 13, 43, 47, 50, 52, and 60 and the muscle promoter [3].

Results

Clinical study

Exertional rhabdomyolysis with myoglobinuria was observed in three patients (patients 1, 8, 14). The remaining 12 had myalgias, occasionally associated with cramps (patients 2, 3, 5, 9, 11–13) or with post-exercise muscle stiffness (patients 7, 10, 15). The age range at diagnosis was 17–58 years (mean 29.5 years). All were sporadic cases. Neurological examination was normal in 13 patients and showed moderate calf hypertrophy in 2 (patients 1, 10). Dilated cardiomyopathy was seen in 3 patients (patients 3, 6, 13). Electromyograms revealed a my-

Table 1 Dystrophin expression and clinicopathological features of the five patients with exercise intolerance-dystrophinopathy syndrome (*CK* creatine kinase, *EMG* electromyogram, *PCR* polymerase chain reaction, + present, – absent, *Irr* irregular, *M* male, *Myo* myogenic pattern, *MW* molecular weight, *N* normal, *Y* years, ↑ increased in intensity, ↓ decreased in intensity)

Patients	1	3	8	11	14
Clinical features					
Sex/Age	M/21 Y	M/19 Y	M/18 Y	M/30 Y	M/22 Y
Rhabdomyolysis	+	–	+	–	+
Cramps and Myalgias	–	+	–	+	–
Calf hypertrophy	+	–	–	–	–
Cardiopathy	+	–	–	–	–
CK/EMG	↑/N	↑/N	↑/Myo	↑/N	↑/N
Muscle biopsy					
Fiber size variation	+	+	+	+	+
Internal nuclei	+	+	+	+	+
Nerotic and regenerative fibers	+	+	+	+	–
Immunohistochemistry					
Dys 1	–	–	Irr	–	Irr
Dys 2	N	Irr	Irr	Irr	Irr
Dys 3	Irr	–	Irr	Irr	Irr
Immunoblot					
Dys 1	–	–	–	–	–
Dys 2	↓	↓	↓	↓ Low WM	N
Dys 3	↓	↓	↓	↓ Low WM	↓
DNA analysis					
Multiplex PCR	N	Deletion exons 45–52	N	Deletion exons 13–19	N

opathic pattern in two patients (patients 6, 8) and were normal in 13. The CK serum level was normal in 7 patients and increased in 8 (patients 1, 3, 4, 6, 8, 11, 14, 15). The results are summarized in Table 1 for patients 1, 3, 8, 11 and 14.

Histoenzymology and immunohistochemistry

Histoenzymology

A mild myopathic pattern (a few necrotic and regenerating fibers, centralizations, and splitting) was seen in the muscle biopsy samples from five patients (patients 1, 3, 8, 11, 14, Table 1). Minor and nonspecific changes were observed in patients 6, 7, 9, 10 and 15; the muscle biopsy specimens for the remaining five patients (patients 2, 4, 5, 12, 13) appeared normal. Endomysial collagen was not increased and there was no adipose tissue proliferation.

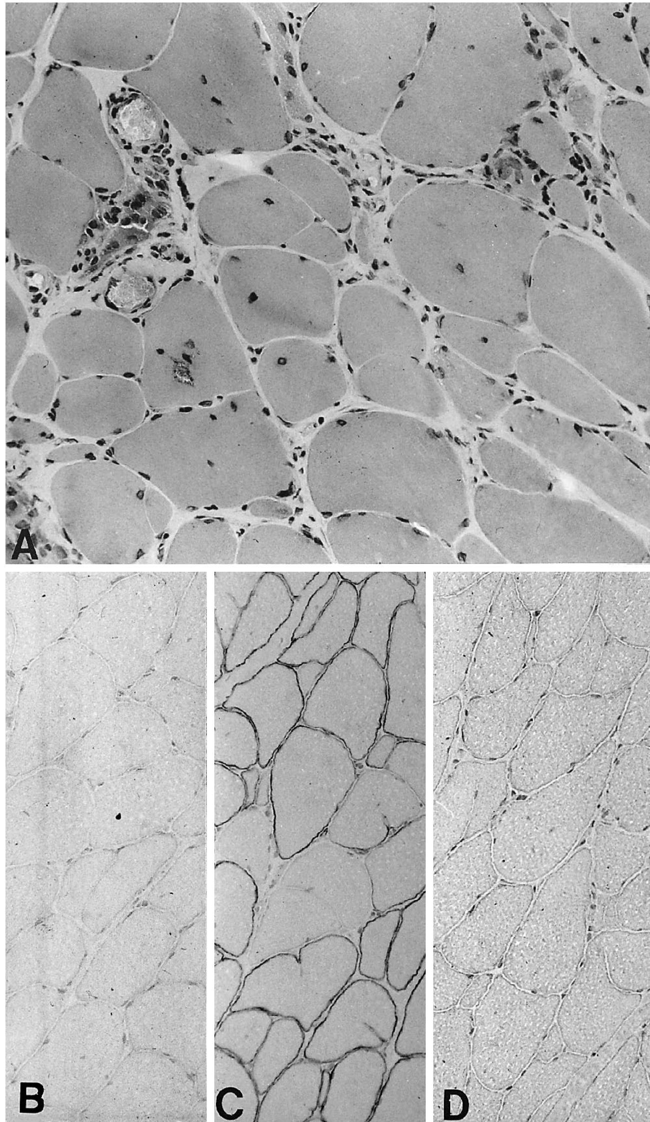

Fig. 1 Pathological changes and immunohistochemical detection of dystrophin in patient 3, showing fiber size variation, fiber splitting, internal nuclei and rare necrotic and regenerative fibers (**A**, hematoxylin-eosin), lack of anti-Dys 1 immunoreactivity (**B**), variation in labeling intensity with anti-Dys 2 (**C**), and lack of anti-Dys 3 immunoreactivity (**D**). **A**–**D** Serial sections; **A** × 190, **B**–**D** × 120

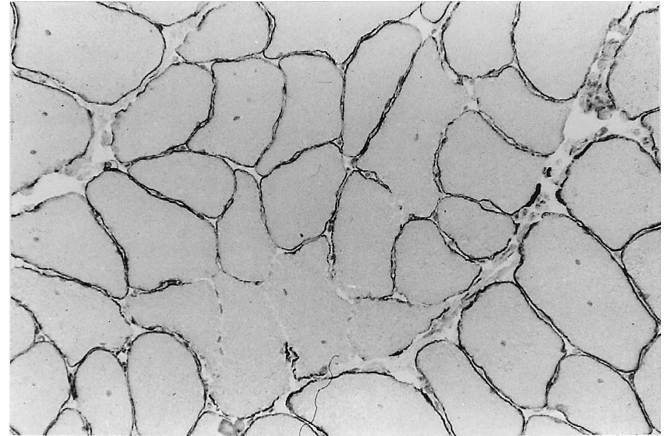

Fig. 2 Pathological changes and immunohistochemical detection of dystrophin in patient 8: anti-Dys 2 immunoreactivity shows a mosaic pattern. × 200

Immunohistochemistry (Figs. 1, 2)

Immunoreactivity was classified as irregular when diffuse weak immunostaining together with intrafiber variation in labeling intensity was observed. Anti-Dys 1 immunoreactivity was lacking in patients 1, 3 and 11, irregular in patients 4, 5, 8–10, 12 and 14, and normal in others. Anti-Dys 2 immunostaining was irregular in patients 3, 8, 11 and 14 and normal in others. Anti-Dys 3 was absent in patient 3, and irregular in patients 1, 2, 4, 6–8 and 11–15. Interestingly, patient 8 shows a mosaic expression of fibers negative (10%) and positive (90%) with all anti-dystrophin antibodies.

In contrast, loss of anti-Dys 2 immunoreactivity was observed in DMD muscles and anti-dystrophin immunoreactivity was normal in control muscles. Anti-spectrin immunoreactivity was normal in all muscles. Immunostaining was performed twice for each muscle specimen and the same results were observed. With the anti-utrophin antibody, the sarcolemma of all muscle fibers was strongly labeled in patient 1, but weakly labeled in patients 8 and 11. Utrophin was not expressed in the sarcolemma of muscles from the other two patients tested (patients 12, 15).

Immunoblot analysis

Immunoblot analysis was performed for the 15 selected patients and for one normal control muscle. The procedure was performed twice for each antibody and gave the same results.

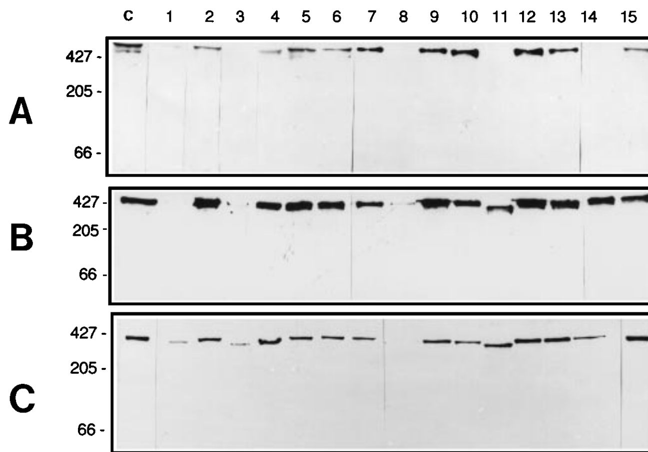

Fig. 3A–C Immunoblot analysis of dystrophin from normal muscle (*C*), and patients 1–15, respectively. **A** Immunoblot analysis of Dys 1, showing lack of Dys 1 expression in patients 1, 3, 8, 11 and 14. **B** Immunoblot analysis of Dys 2, showing weak Dys 2 expression in patients 1, 3 and 8, and dystrophin of low molecular weight in the patient 11; normal expression is seen in other patients. **C** Immunoblot analysis of Dys 3, showing weak Dys 3 expression in patients 1 and 3, and an almost total lack of Dys 3 expression in patient 8. Dys 3 reveals dystrophin of low molecular weight in patient 11

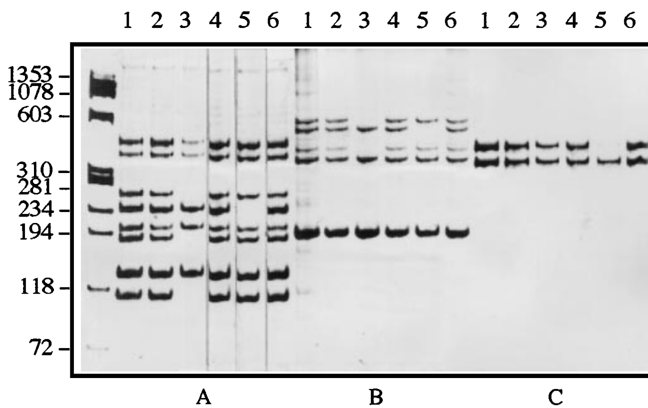

Fig. 4A–C Polymerase chain reaction (PCR) analyses of the dystrophin gene. *Lane 1* Normal muscle, *lane 2* patient 1, *lane 3* patient 3, *lane 4* patient 8, *lane 5* patient 11, *lane 6* patient 14. **A** PCR analysis of exons 3, 43, 50, 13, 6, 47, 60, 52 (from *top* to *bottom*). Note that exons 50, 47, 52 are missing in patient 3 (*lane 3*), and that exon 13 is missing in patient 11 (*lane 5*). **B** PCR analysis of exons, 45, 19, 51, 12, 4 (from *top* to *bottom*). Exons 45 and 51 are deleted in patient 3 (*lane 3*), and exon 19 in patient 11 (*lane 5*). **C** PCR analysis of exons 17 and 8 (from *top* to *bottom*). Exon 8 is lacking in patient 11 (*lane 5*)

Immunoblot analysis was abnormal for patients 1, 3, 8, 11 and 14. Anti-Dys 1 immunoreactivity was almost absent in these patients (Fig. 3A). Anti-Dys 2 immunoreactivity was reduced in intensity in patients 1, 3 and 8, whereas a dystrophin molecule of reduced molecular size was seen in patient 11 (Fig. 3B). With anti-Dys 3 antibody, immunoreactivity was reduced in intensity in patients 1, 3 and 14 and barely detectable in patient 8. A band of reduced molecular size but normal intensity was observed in one muscle extract (patient 11; Fig. 3C). The remaining patients showed normal anti-dystrophin immunoreactivity (intensity and molecular size) as compared to the control muscle (Fig. 3).

DNA analysis

Multiplex PCR showed the presence of a deletion in the dystrophin gene in two patients only (patient 3 and 11; Fig. 4). A lack of exons 45, 47, 50–52 was observed in patient 3 and a lack of exons 13, 17, 19 in patient 11. Exons 44 and 60 were present in patient 3. The exact size of the deletion is still unknown, but affects at least exons 45–52. Exon 12 was present in patient 12, but since multiplex PCR does not explore exons 20–42 (mid rod domain), we cannot determine the exact size of the deletion, although it spans at least exons 13–19.

Discussion

Development of specific monoclonal antibodies directed against the dystrophin molecule and the use of dystrophin expression detected by immunohistochemistry as a diagnostic marker for DMD and BMD have been well established in a series of studies [2, 15, 16, 20]. In DMD, a complete lack of dystrophin from skeletal muscles is pathognomonic, whereas anti-dystrophin immunostaining of BMD muscle reveals discontinuous “patchy” staining [16] and also intrafiber variation in labeling intensity [2]. A few reports have described a new dystrophinopathy phenotype represented by exercise intolerance associated with myalgias, muscle cramps or myoglobinuria, without weakness [4, 9, 12, 13, 17, 19, 21]. In these cases, except in the study performed by Samaha and Quinlan [21], DNA studies have shown deletions in the dystrophin gene. Immunohistochemistry was performed by several groups [12, 17, 19]. Using the anti-60 kDa antibody described by Hoffman et al. [15], Gold et al. [12] observed a slightly fainter staining intensity for dystrophin in three patients compared to normal controls. They thought that, with the methods used, immunohistochemistry did not disclose any clear pathological results. Immunohistochemistry performed using the antibodies described in the present study was normal in one case [17] but showed a mosaic pattern in another [21].

In this report, we have searched for abnormal dystrophin expression using immunohistochemistry, immunoblot and DNA analysis in a series of 15 patients presenting with exercise intolerance and non-informative muscle biopsy. Metabolic disorders, which are the major cause of exercise intolerance, were first excluded by measurement of CPT, and glycolytic and muscle respiratory chain enzymes in muscles.

This report shows that the use of immunohistochemistry with antibodies directed against various regions of the dystrophin – anti-Dys 1 (rod domain), anti-Dys 2 (C terminus) and anti-Dys 3 (N terminus) – is of the utmost importance for the diagnosis of the exercise intolerance-

dystrophinopathy syndrome. Absence of staining in the rod domain using immunohistochemistry provides a secure diagnosis of the syndrome (present series, patients 1, 3, 11). In addition, if immunohistochemistry shows abnormal staining (irregular pattern but not absence) with the three antibodies, diagnosis is reasonably secure but should be confirmed by immunoblot analysis (present series, patients 8 and 14, and patient reported by Malapert et al. [19]). In only one case, however, was the immunohistochemistry performed with these antibodies normal [17].

Immunohistochemical and Western blot analysis of dystrophin are more useful for the diagnosis than DNA analysis using the multiplex PCR technique. This approach is interesting for a rapid screening of dystrophin gene deletions in BMD and DMD [3, 7]. Using this technique, a dystrophin gene deletion was only detected in patients 3 and 11. As suggested by the immunohistochemical pattern, patient 8 may have a somatic mosaicism, and the lack of DNA deletion is consistent with this hypothesis. The remaining patients may have either a deletion in the rod domain, not explored by the multiplex PCR (from exon 20 to 42), or a point mutation. The immunohistochemical and immunoblot analyses using anti-Dys 1 antibody indicate that the rod domain is mainly involved in the exercise intolerance-dystrophinopathy syndrome.

It is likely that, when anti-dystrophin immunostaining is weak or absent, immunohistochemical detection of utrophin (dystrophin-related protein [18]) would be useful for diagnosis if it shows abnormal expression on the sarcolemma. Analysis of anti-utrophin immunoreactivity was only performed for a few muscle specimens of our series (from patients 1, 8, 11, 13, 15). All muscle fibers were strongly labeled with anti-utrophin antibody in patient 1, but only weakly in patients 8 and 11. Utrophin was not expressed in the sarcolemma in muscles from the other patients tested (patients 12 and 15).

The present study and a review of the literature [4, 9, 12, 13, 17, 19, 21] show that the exercise intolerance-dystrophinopathy syndrome displays characteristic clinical, biological and histopathological features. Clinically, it only affected males (except for the case reported by Malapert et al. [19]), before 33 years of age. Although some reports clearly showed X-linked inheritance [12, 13], other cases were sporadic ([4, 9, 17, 19, 21] and present series). All patients presented with exertional rhabdomyolysis or cramps but no weakness. It is worth noticing that in our series, dystrophinopathy was not observed in patients presenting with myalgias only. Some patients also exhibited calf hypertrophy ([13, 19] and patient 1 of the present study) or cardiomyopathy (patients 1 and 2 of [13]; and patient 3 of the present study). The CK level was always abnormal at rest and histopathology revealed mild myopathic changes in all patients. Some patients reported by Samaha and Quinlan [21] showed normal muscle biopsy samples and normal CK levels at rest; however, for this series, immunohistochemistry was not performed, DNA analysis was always normal and diagnosis of dystrophinopathy was performed on Western blot analysis using only one anti-dystrophin antibody.

The exercise intolerance-dystrophinopathy syndrome is usually associated with a deletion in the dystrophin gene that may affect various parts of the rod domain; the same deletion has been previously reported in patients with typical BMD or DMD [3].

In conclusion, besides metabolic disorders, dystrophinopathy is one etiology of idiopathic myoglobinuria, requiring genetic counseling. Moreover, if patients fulfill the outlined criteria for the exercise-intolerance dystrophinopathy syndrome, it would be cost effective to search for abnormal dystrophin expression by immunohistochemistry using antibodies directed against the various regions of the protein before screening for metabolic disorders. The actual incidence of cramps and myalgia cases with dystrophinopathy remains to be determined.

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