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Assessment of the 50-kDa dystrophin-associated glycoprotein in Brazilian patients with severe childhood autosomal recessive muscular dystrophy

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Abstract

Recently, we have demonstrated the specific deficiency of the 50-kDa dystrophin-associated glycoprotein (50DAG) in severe childhood autosomal recessive muscular dystrophy with Duchenne-like phenotype (SCARMD or AR-DLMD), a disease first reported in Tunisia and now presumed to be prevalent in North Africa and the Middle East. Here we demonstrate the deficiency of the 50DAG in one caucasoid and 5 negroid Brazilian patients with severe muscular dystrophy, which confirms that AR-DLMD with the 50DAG deficiency is not confined to the Arab populations. Without the analysis of both dystrophin and 50DAG, isolated male patients with this condition could be undiagnosed or misdiagnosed as having Duchenne or severe Becker muscular dystrophy. We also report, for the first time, the normal expression of the 50DAG and other dystrophin-associated proteins in one negroid and 2 caucasoid Brazilian patients with a phenotype indistinguishable from that of AR-DLMD with 50DAG deficiency. This is consistent with the genetic heterogeneity for the phenotype of AR-DLMD.

Key words: Severe childhood autosomal recessive muscular dystrophy; Dystrophin-glycoprotein complex; 50-kDa dystrophin-associated glycoprotein

1. Introduction

Duchenne muscular dystrophy (DMD) is a lethal neuromuscular disease caused by the absence of dystrophin (Hoffman et al. 1988). Dystrophin is associated with a large oligomeric complex of sarcolemmal glycoproteins, including dystroglycan which binds the extracellular matrix component, laminin (Ervasti et al. 1990; Ibraghimov-Beskrovnaya et al. 1992). These findings indicate that the dystrophin-glycoprotein complex spans the sarcolemma to provide a linkage between the subsarcolemmal cytoskeleton and the extracellular matrix. In DMD, the absence of dystrophin leads to a great reduction in all of the dystrophin-associated proteins, thus causing the disruption of this linkage (Ervasti et al. 1990; Ibraghimov-Beskrovnaya et al. 1992; Ohlendieck et al. 1993). The resulting instability of the sarcolemma is presumed to render muscle cells susceptible to necrosis.

A severe form of autosomal recessive muscular dystrophy, affecting both sexes equally, clinically indistinguishable from Xp21 muscular dystrophy was first reported in Tunisia (Ben Hamida et al. 1983). This condition has been referred to as severe childhood autosomal recessive muscular dystrophy (SCARMD) or autosomal recessive Duchenne like muscular dystrophy (AR-DLMD). Because dystrophin-associated proteins are deficient in Duchenne muscular dystrophy, it seemed possible that a primary defect in one of the dystrophin-associated proteins could be the cause of autosomal muscular dystrophy. Recently, we have demonstrated the specific deficiency of the 50-kDa dystrophin-associated glycoprotein (50DAG) in one

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Lebanese and 3 Algerian SCARMD patients (Matsumura et al. 1992). The deficiency of the 50DAG is thought to lead to muscle cell necrosis in SCARMD patients (Matsumura et al. 1992).

Since the Brazilian population has a high rate of racial miscegenation, it is important to determine if a deficiency of 50DAG is also observed in Brazilian patients with the AR-DLMD phenotype (Zatz et al. 1989) or if there is genetic heterogeneity as was recently found for the adult forms of autosomal recessive limb-girdle muscular dystrophy (Passos-Bueno et al. 1993a). In order to answer these questions, which are fundamental for accurate diagnosis, interpretation of linkage analysis and potential future therapies, we investigated the status of the components of the dystrophin-glycoprotein complex in patients afflicted with AR-DLMD in the Brazilian population.

2. Methods

Patients

A total of 10 patients with AR-DLMD (3 males and 7 females from nine unrelated families) were selected as potential candidates for the deficiency of the 50DAG (Table 1). Clinical details of these patients are described below. Two boys with DMD, 2 female patients with LGMD (one linked to chromosome 15q and the other not linked to chromosome 15q (Passos-Bueno et al. 1993a)) and one normal human were included as controls in the study. All were screened in the Centro de Miopatias, Instituto de Biociências, Universidade de São Paulo. Above diagnosis was established based on clinical findings, family history, grossly elevated serum creatine kinase (CK) value, electromyography, DNA analysis of the dystrophin gene, muscle histology and dystrophin tests (immunohistochemistry and immunoblotting). None of the non-DMD patients had deletions in the dystrophin gene by means of polymerase chain reaction (PCR) using 18 pairs of primers and Southern blot analysis.

2.1. Case reports

Patient 1

This 10-year-old negroid boy, who had been reported previously (Vainzof et al. 1991), started walking at 18 months of age. He had difficulty in climbing stairs and fell frequently since the age of 5 years. Calf hypertrophy was noted at the age of 7 years. He was confined to a wheelchair at the age of 9 years. Serum CK value was increased 140-fold of the normal upper-limit value at 7 years of age but only 5-fold at 10 years. The proband's parents are first-degree cousins. He has an older sister (aged 11 years) and a younger brother (aged 5 years) who are both normal.

Patient 2

This 8-year-old negroid boy was referred to us because of frequent falls and difficulty in running and climbing stairs since the age of 4 years. At physical examination, he showed calf hypertrophy, positive Gowers' sign and pronounced scoliosis. His serum CK value was increased 25-fold of the normal upper-limit value. He died suddenly, at the age of 9 years, apparently of heart failure. The proband's family is highly inbred and his parents are first-degree cousins. He had seven affected relatives (5 girls and 2 boys) all with severe progression. Four of them died between 16 and 25 years of age. The three who are still alive (currently aged 16, 18 and 21 years) were confined to a wheelchair between 10 and 14 years of age.

Patient 3

This 14-year-old negroid boy was first seen by us at the age of 8 years. He had calf hypertrophy and complaints of frequent falls and difficulty in climbing stairs since 7 years of age. His parents are half-first-degree cousins (the proband's grandfather and his grandmother are half sibs). He has an older sister (aged 10 years) and a younger brother (aged 7 years), both normal. His condition deteriorated rapidly and he was confined to a wheelchair at the age of 14 years. Repeated serum CK tests showed a 150-fold increase of the

Table 1
Summary of patients' data

Patient No.	Sex	Age (yrs)	Onset (yrs)	Wheel-chair [a]	Death	Calf hypertrophy	CK [b] (age)	Consanguinity	Race [c]	50DAG [d]
1	M	10	5	9	–	+	140 (7y)	yes	N	–
2	M	8	4	–	9y	+	25 (8y)	yes	N	–
3	M	14	7	14y	–	+	150 (8y)	yes	N	–
4	F	16	9	–	–	+	28 (15y)	yes	N	–
5	F	13	6	11y	–	+	20 (11y)	no	N	–
6	F	17	8	14y	–	+	8 (17y)	no	C	–
7	F	12	4	–	–	+	50 (4y)	no	N	+
8 [e]	F	13	4	–	–	+	27 (11y)	no	C	+
9 [e]	F	6	3	–	–	+	60 (3y)	no	C	+
10	F	6	2	–	–	–	12 (6y)	no	C	+

[a] Age when the patient was confined to a wheelchair.
[b] Increase of serum creatine kinase value over the normal value (normal upper limit = 1).
[c] Racial background; N, negroid; C, caucasoid.
[d] Status of the 50DAG in skeletal muscle: – = deficiency; + = normal.
[e] Patients 8 and 9 are sisters.

normal upper-limit value at 8 years of age but of only 11-fold at 14 years.

Patient 4

This 16-year-old negroid girl was first seen in our center at the age of 15 years. The parents refer onset of symptoms at 9 years of age. At the first physical examination, she had calf hypertrophy and was unable to rise from the floor or chair without help. Her parents, who are first-degree cousins, had a total of 10 children. The proband has an affected brother (aged 19 years) and an affected sister (aged 18 years), who were confined to a wheelchair at the age of 16 and 14 years, respectively, and who are currently severely disabled. Serum CK value was increased 28-fold of the normal upper-limit value in the proband and 5- and 10-fold in the affected brother and sister, respectively.

Patient 5

This 13-year-old negroid girl was first seen by us at the age of 12 years. Her first complaint, at 6 years of age, was difficulty in walking and running. She was confined to a wheelchair at 11 years of age and currently she has difficulty in raising her arms. Her serum CK value at 11 years of age was increased 20-fold of the normal upper-limit value. Her parents are non-consanguineous.

Patient 6

This 17-year-old caucasoid girl was first seen by us at the age of 15 years. She fell frequently and had difficulty in running and climbing stairs since 8 years of age. She was confined to a wheelchair at the age of 14 years. Her serum CK value was increased 6-fold of the normal upper-limit value at 15 years of age and 8-fold at 17 years. She has five normal siblings and two affected brothers, who are currently 21 and 19 years old, both confined to a wheelchair since the age of 11 years. Her parents are non-consanguineous.

Patient 7

This 12-year-old negroid girl was first seen by us when she started walking at 15 months of age. At that time her serum CK was increased 50-fold of the normal upper-limit value. At the age of 4 years, she had calf hypertrophy, difficulties in running and climbing stairs, and positive Gowers' sign. She is still ambulant but her 2 older affected sisters were confined to a wheelchair at the age of 10 and 12 years, respectively. Both of these affected sisters had calf hypertrophy and grossly elevated serum CK value at the early stages of the disease (up to 92-fold of the normal upper-limit value). One of them died suddenly at the age of 14 years. The parents are non-consanguineous.

Patients 8 and 9

These 2 patients are sisters of caucasoid origin. Patient 8, a 13-year-old girl, was first seen by us at the age of 11 years. She had calf hypertrophy, walked on her toes and complained of difficulties in running and climbing stairs since the age of 4 years. She is still ambulant but unable to rise from a chair without support. Her serum CK value was increased 27-fold of the normal upper-limit value at the age of 11 years.

Patient 9, a 6-year-old sister of patient 8, was first examined by us at the age of 3 years. She had calf hypertrophy and grossly elevated serum CK value (60-fold of the normal upper-limit value). However, she had no symptoms except complaints of pain in her legs. Currently, she has difficulties in running, falls frequently and has positive Gowers' sign. Their parents are non-consanguineous and have a 7-year-old normal girl in addition to the affected sisters.

Patient 10

This 6-year-old caucasoid girl was referred to us due to pronounced muscle weakness since the age of 2 years. At physical examination, she had difficulties in climbing stairs, positive Gowers' sign and complaints of pain in her legs and frequent falls. She had no calf hypertrophy and her serum CK value was elevated to 12-fold of the normal upper-limit value. She has a 7-year-old normal brother. The parents are non-consanguineous.

2.2. Immunohistochemistry

As screening tests, dystrophin was studied by both immunofluorescence and immunoblot analyses, using at least two antibodies against dystrophin (N- and C-terminal regions), as reported previously (Vainzof et al. 1991). With the exception of the DMD boys who lacked dystrophin and of the two patients (patients 3 and 9, Table 1) who showed patchy dystrophin staining on immunofluorescence and dystrophin of normal size but with reduced quantity on immunoblot analysis, all others showed a normal dystrophin pattern.

Indirect immunofluorescence microscopy of 7-μm cryosections from skeletal muscle biopsy specimens was performed as described previously (Ohlendieck et al. 1993; Matsumura et al. 1992). Monoclonal antibody $VIA4_2$ against dystrophin and $IVD3_1$ against the 50DAG were characterized previously (Ervasti et al. 1990; Ohlendieck et al. 1993; Matsumura et al. 1992). Specific antibodies against the 156DAG, 59DAP, 43DAG and 35DAG were affinity-purified as described (Ervasti et al. 1990; Ibraghimov-Beskrovnaya et al. 1992; Ohlendieck et al. 1993; Matsumura et al. 1992). Blocking was performed by a 1-hr incubation with 5% bovine serum albumin in PBS (50 mM sodium phosphate, pH 7.4, 0.9% NaCl). Incubation with primary antibodies was performed for 2 h. In the case of monoclonal antibodies, cryosections were incubated with 1:200 diluted fluorescein-labeled goat anti-mouse IgG (Boehringer-Mannheim) for 1 h. In the case of sheep primary antibodies, cryosections were incubated for 1 h with 1:500 diluted biotinylated rabbit anti-sheep IgG (Vector Laboratories) followed by incubation for 30 min with 1:1000 diluted fluorescein-conjugated streptavidin (Jackson ImmunoResearch Laboratories). Incubation with all antibodies was performed at room temperature. Each incubation was followed by washing with PBS. Final specimens were examined under a Zeiss Axioplan fluorescence microscope. For reliable comparison, cryosections from different patients were placed on the same microscopy slide and processed identically. In addition, photographs were taken under identical conditions with the same exposure time.

3. Results

Antibodies against dystrophin and the dystrophin-associated proteins stained the sarcolemma in skeletal

muscle from normal humans as described previously (Ohlendieck et al. 1993; Matsumura et al. 1992) (Fig. 1). In skeletal muscle from DMD patients, the sarcolemma was not stained by the antibody against dystrophin and the staining for all of the dystrophin-associated proteins was greatly reduced in the sarcolemma (Fig. 1).

In skeletal muscle from the six patients with AR-DLMD (patients 1–6), antibodies against dystrophin, the 156-kDa α-dystroglycan (156DAG), the 59-kDa dystrophin-associated protein (59DAP) and the 43-kDa β-dystroglycan (43DAG) stained the sarcolemma, but staining by antibody against the 50DAG was drastically reduced and almost undetectable (Figs. 1 and 2). The reduction of the 50DAG-staining in these patients was more severe than in DMD patients (Fig. 1). Immunostaining for the 35-kDa dystrophin-associated glycoprotein (35DAG) was slightly to moderately reduced in these six patients compared to normal humans (not shown). On the other hand, in skeletal muscle from the other 4 patients with AR-DLMD (patients 7–10), as well as in the two with LGMD, staining for all the components of the dystrophin-glycoprotein complex was well preserved and indistinguishable from normal humans (Figs. 1 and 2).

4. Discussion

Based on a study of 470 families with patients affected by severe childhood muscular dystrophy, we have estimated as 6.8% the proportion of families with DMD-like phenotype inherited as an autosomal recessive trait (AR-DLMD) in the Brazilian population (Zatz et al. 1989), which is significantly less than the 36.3% found in the Arab population (Farag and Teebi 1990).

The clinical picture of the Brazilian patients with AR-DLMD is very similar to the severe or intermediate forms of Xp21 DMD with little intrafamiliar variability. Most of the patients are confined to a wheelchair from 9 to 16 years of age and usually die in the second or third decade. Serum creatine kinase (CK) levels are grossly elevated and may reach the same range as that observed in young Xp21 DMD patients (Zatz et al. 1991). Histopathological as well as electromyography findings show a severe myopathic pattern, indistinguishable from that of DMD.

Here we have demonstrated the specific deficiency of the 50DAG in one caucasoid and 5 negroid Brazilian patients (patients 1–6, Table 1) with AR-DLMD. One interesting finding was the 50DAG deficiency in a male sporadic patient with AR-DLMD (patient 3, Table

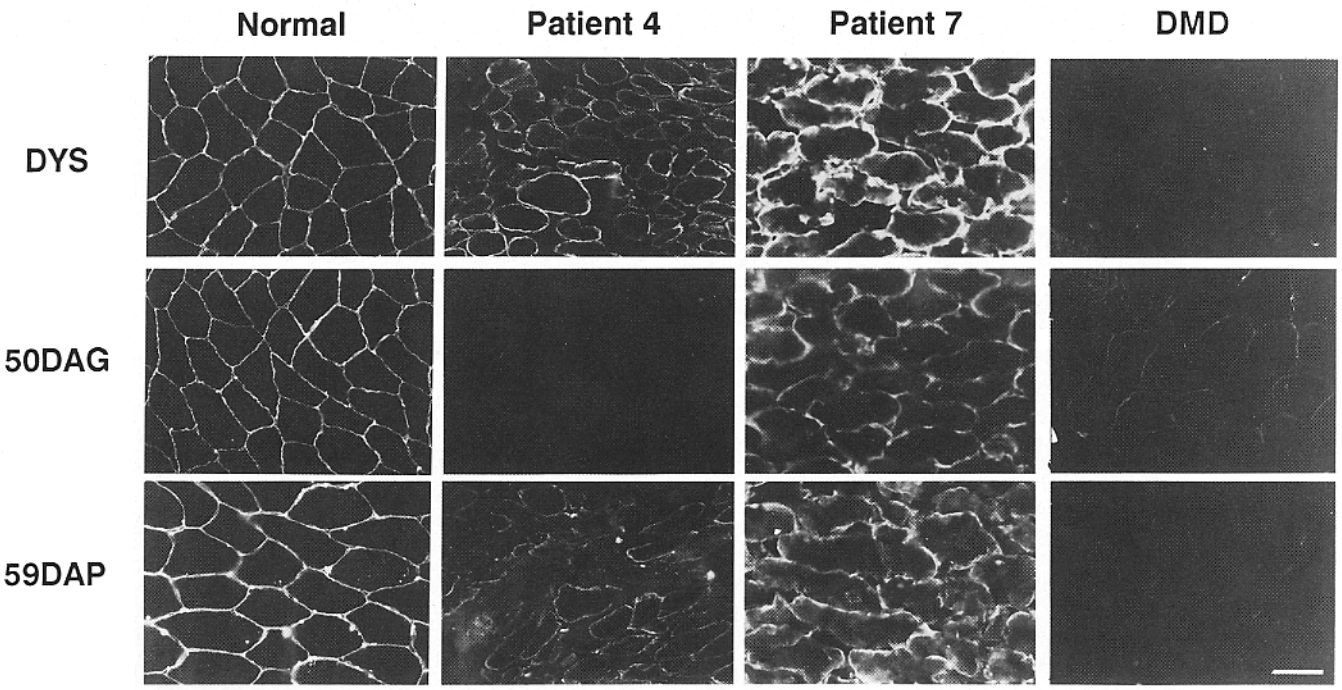

Fig. 1. Immunohistochemical analysis of dystrophin and the dystrophin-associated proteins in biopsied skeletal muscle from a normal human, a DMD patient, and patients 4 and 7 (Table 1). Transverse cryosections (7 μm) were immunostained with antibodies against dystrophin, 59DAP and 50DAG. In patient 4, the 50DAG was undetectable, while dystrophin and the 50DAP were preserved in the sarcolemma. In patient 7, all of these proteins were well preserved in the sarcolemma despite the distorted morphology of the muscle fibers due to freezing artifacts. In the dystrophin-deficient DMD patient, the 59DAP and 50DAG were greatly reduced but still barely detectable in the sarcolemma. Bar = 50 μm.

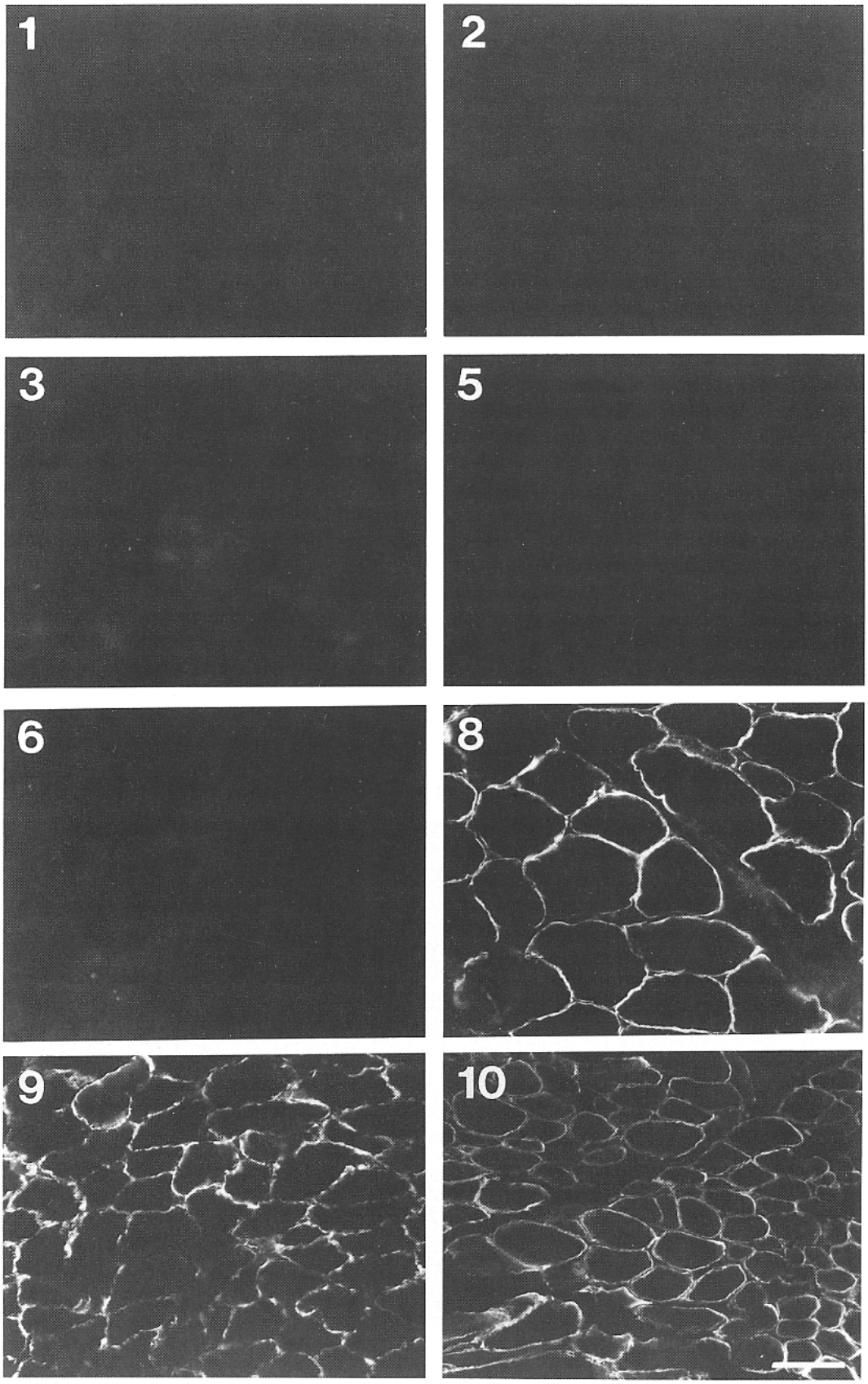

Fig. 2. Immunohistochemical analysis of the 50DAG in biopsied skeletal muscle from patients 1, 2, 3, 5, 6, 8, 9 and 10 (Table 1). Transverse cryosections (7 μm) were immunostained with antibody against the 50DAG. In patients 1, 2, 3, 5 and 6, the 50DAG was undetectable, while the 50DAG was well preserved in the sarcolemma of patients 8, 9, and 10. Bar = 50 μm.

1). Based on the results of dystrophin testing, which showed a patchy dystrophin staining by immunohistochemistry and normal-sized dystrophin of reduced quantity by immunoblot analysis (not shown), this male patient could have been misdiagnosed as severe Becker muscular dystrophy (Hoffman et al. 1988). This observation reinforced the importance of testing the 50DAG, in addition to dystrophin (as previously suggested by us, Vainzof et al. 1991), in all patients with DMD/BMD phenotype who have no detectable abnormalities of the dystrophin gene and in whom X-linked inheritance cannot be confirmed.

On the other hand, 4 patients with similar AR-DLMD phenotype (patients 7–10, Table 1) showed no deficiency of the 50DAG. The clinical picture of patient 10 seems to be slightly different from that of AR-DLMD: this 6-year-old girl had very early onset of symptoms and serum CK value which was below the range of the DMD patients of comparable age, although she had pronounced muscle weakness and severe myopathic pattern on muscle histology. However, the three other female patients (patients 7–9, Table 1) had a typical clinical picture of AR-DLMD. Patients 8 and 9 are sisters and have non-consanguineous caucasoid parents. Patient 7 and her two older affected sisters are from a negroid family with no consanguinity. These three siblings, who had been followed by us for many years, had a severe phenotype indistinguishable from that of AR-DLMD with the 50DAG deficiency. These results suggest the heterogeneity for the phenotype of AR-DLMD at the protein level, one with the 50DAG deficiency and the other(s) with normal 50DAG, although a possibility exists that the apparently normal 50DAG observed by immunohistochemistry could be functionally defective.

In a recent publication, Ben Othmane et al. (1992) reported linkage of Tunisian SCARMD to the pericentromeric region of chromosome 13q. Subsequently, a deficiency of 50DAG was found in Algerian patients linked to 13q (Azibi et al. 1993). In order to verify if the Brazilian patients with the 50 DAG deficiency are caused by a defective gene in the same locus, linkage analysis was performed with 13q markers.

Four AR-DLMD families, large enough for linkage analysis, who had been previously excluded from the 15q region (Passos-Beuno et al. 1993b) were selected. In three of them, affected patients were deficient for 50DAG (patients 2,4, and 6, Table 1), and in one family 50DAG analysis was normal (patient 7). Results of linkage analysis excluded the 13q region as a candidate gene in the four families (Passos-Bueno et al. 1993c). These observations indicate that their condition is not caused by the same genetic defect as in patients who were found to be linked to 13q, thus confirming genetic heterogeneity of AR-DLMD at the DNA level.

In summary, the present data as well as recent data on linkage analysis suggest genetic heterogeneity within both (1) AR-DLMD phenotype, and (2) the AR-DLMD with the deficiency of the 50DAG.

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Note added in proof (received 18 March 1994)

While this manuscript was under review, the cDNA encoding 50DAG, which is now known as "adhalin," was cloned from rabbit skeletal muscle (Roberds, S.L., Anderson, R.D., Ibraghimov-Beskrovnaya, O. and Campbell, K.P. (1994) Primary structure and muscle-specific expression of the 50-kDa dystrophin-associated glycoprotein (adhalin). J. Biol. Chem., 268; 23739–23742).

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