

# Clinical Heterogeneity of Adhalin Deficiency

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**We report adhalin deficiency in 8 patients with clinically diagnosed muscular dystrophy, dystrophic histopathological features, high plasma creatine kinase levels, normal expression of dystrophin, and marked variability of symptoms. Although the distribution of hyposthenia was similar in all 8 patients and predominantly involved muscles in the pelvic girdle, age at onset and rate of disease progression were highly variable: In 2 patients onset, at ages 24 and 25, was later than has been previously observed. We found no apparent relation between disease severity and the quantity of adhalin expressed. Two kinds of myopathy with adhalin deficiency have been reported: one caused by a mutation in the adhalin gene on chromosome 17 (primary adhalinopathy) and the other linked to chromosome 13. The product of the gene on chromosome 13 is probably associated with adhalin and its deficiency results in secondary adhalinopathy. The severity of clinical phenotypes in these adhalinopathies seems to relate more to the kind and site of the mutations than to the residual amount of the protein. We also detected a variable reduction in the laminin  $\beta$ 1 subunit by immunohistochemistry in most patients, confirming that this is commonly associated with adhalin deficiency.**

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Dystrophin, the protein product of the Duchenne muscular dystrophy (DMD) locus, is tightly associated through its cysteine-rich and C-terminal domains to a large glycoprotein complex. This dystrophin-glycoprotein complex (DGC) is composed of at least five transmembrane proteins (50-kd adhalin, 43-kd  $\beta$ -dystroglycan, 43-kd dystrophin-associated glycoprotein [DAG] or A3b, 35-kd DAG, and 25-kd dystrophin-associated protein [DAP]), one extracellular protein (156-kd  $\alpha$ -dystroglycan), and four cytoplasmic proteins (syn-trophin triplet and dystrophin) [1-10]. In skeletal muscle, interactions between  $\alpha$ -dystroglycan and merosin as well as between dystrophin and cytoskeletal actin filaments have been identified, indicating that one function of the DGC is to provide a link between the extracellular matrix and the cytoskeleton [6, 7, 11, 12]. Immunological investigation has shown that the DAPs are greatly reduced in the skeletal muscle of DMD patients and the mdx mouse [1, 13, 14].

Recently adhalin deficiency was found in severe childhood autosomal recessive muscular dystrophy (SCARMD), which has a DMD-like phenotype [15]. This disease affects both males and females, was first identified in Tunisian families [16], and has since been found in other populations.

By linkage analysis in North African families the SCARMD locus was mapped to chromosome 13q12 [17, 18], while the adhalin gene itself was mapped to chromosome 17q12-q21.33 [19, 20], excluding its direct involvement in 13q12-linked SCARMD. However, defects in the adhalin gene have now been linked to autosomal recessive muscular dystrophies in families from France, Germany, and Italy, as well as North Africa [20, 21].

Originally adhalin deficiencies were observed in clinically severe phenotypes resembling DMD [15], and later, more heterogeneity of clinical expression was described [21-24]. We now report 8 patients with adhalin deficiency among 86 patients with clinically diagnosed muscular dystrophy, with dystrophic histopathological features, high plasma creatine kinase (CK) levels, and normal expression of dystrophin.

## Materials and Methods

### Patients

The clinical features are summarized in the Table.

Patient 1 was first examined at age 15 for slight muscle weakness. Clinical examination revealed mild weakness of the pelvic girdle, increased lumbar lordosis, and waddling gait. No calf hypertrophy was noted. The plasma CK level

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### Clinical Features

Patient No.	Sex	Age (yr) at Onset	Age (yr) at Diagnosis	Family History	Consanguinity	CK level (units/liter)	Cardiomyopathy	Hypertrophic Calves	Clinical Involvement
1	M	15	18	Yes	No	2,854	Yes	No	Mild
2	M	Neonatal	3	No	Yes	10,000	ND	Yes	Asymptomatic
3	F	24	29	No	No	1,062	No	Yes	Moderate
4	F	25	42	No	No	1,478	No	No	Moderate
5	F	5	10	Yes	No	5,142	No	Yes	Severe
6	F	9	32	Yes	No	277	No	Yes	Severe
7	F	9	27	Yes	No	864	No	Yes	Severe
8	F	9	26	Yes	No	905	No	Yes	Severe

ND = not done.

was 2,854 units/liter (normal value: 25–195 units/liter). Muscle computed tomography (CT) disclosed mild fibrosis. A muscle biopsy specimen, taken when he was 18, showed mild, mainly perimysial connective tissue proliferation, increased variability of fiber diameter, centrally located nuclei, and degenerating fibers. Since then, the condition of the patient slightly deteriorated (he was 25 years old at the time of writing). An echocardiogram revealed dilatative cardiomyopathy and left ventricular hypokinesia.

A paternal female cousin of Patient 1 showed progressive weakness of proximal limb muscles since early childhood and was confined to a wheelchair at age 15. Her parents were second cousins. On consecutive determinations, plasma CK levels were markedly increased (up to 11,000 units/liter). Her karyotype, electrocardiogram (ECG), and echocardiogram were normal. No intellectual impairment was noted. Examination of a muscle biopsy specimen revealed a dystrophic picture with marked fibrosis. The muscle sample from this patient was not suitable for immunochemical analysis.

Patient 2, a 3-year-old boy from a gypsy family resident in northern Italy, whose parents were first cousins, came to our observation for high plasma CK levels (10,000 units/liter). His motor milestones had been normal. Clinical examination showed only hypertrophy of calves. His six brothers and sisters were all normal. A muscle biopsy specimen showed mild endomysial and perimysial connective tissue proliferation, hypercontracted fibers, necrotic fibers, and central nuclei.

Patient 3 is a 33-year-old woman who first complained of weakness at age 24. At age 26 clinical examination showed mild weakness of pelvic girdle muscles and mild hypertrophy of calves; examination of a muscle biopsy specimen revealed mild perimysial connective tissue proliferation and a few necrotic fibers. Limb girdle dystrophy was diagnosed. Three years later, she was seen by an independent neurologist who suspected inflammatory myopathy and requested a second biopsy. This specimen showed a marked increase of endomysial and perimysial connective tissue, marked variability of fiber size, central nuclei, and splittings, confirming the diagnosis of muscular dystrophy. At this point we saw her again; she did not appear worsened. Plasma CK level was 1,062 units/liter.

Patient 4 is a 43-year-old woman who complained of weakness of proximal lower limb muscles since age 25. On

clinical examination at age 42 proximal upper and lower limb muscles were moderately weak. Plasma CK levels varied between 542 and 1,478 units/liter on several determinations. Limb girdle dystrophy was diagnosed and a muscle biopsy performed. The specimen showed marked variability of fiber size, fiber degeneration, central nuclei, and connective tissue proliferation at endomysial and perimysial sites.

Patient 5, a 10-year-old girl, had normal motor milestones and normal muscle strength until age 5, when lower limb weakness became evident. On recent clinical examination she had marked pelvic muscle weakness, waddling gait, and lordosis and could get up from the floor only with Gower's maneuver. The plasma CK level was 5,142 units/liter. Cardiac function was normal. A muscle biopsy specimen showed marked fibrous connective tissue proliferation at endomysial and perimysial sites, hypertrophic and atrophic fibers, and central nuclei. A sister of the patient, aged 13 years, could walk with calipers and had hypertrophic calves and multiple contractures. ECG and echocardiogram were normal; mild restrictive pulmonary function was observed; the CK level was 1,388 units/liter.

Patients 6, 7, and 8 were 3 sisters 32, 27, and 26 years old, respectively. They were all able to walk unaided at 15 months but could never run. Their parents first noticed mild weakness in climbing stairs and getting up from the floor when they were about 9 years old. On clinical examination they had calf hypertrophy and pelvic and proximal lower limb muscular weakness. CK plasma levels were high (Patient 6: 277 units/liter; Patient 7: 864 units/liter; Patient 8: 905 units/liter). Cardiac function was normal; Patient 6 had a mild reduction in vital capacity. Severe progression of muscle weakness was observed in Patient 6, who lost the ability to walk at the age of 29; Patients 7 and 8 could still walk slowly for a short time. Examination of a muscle biopsy specimen from Patient 6 disclosed marked proliferation of perimysial and endomysial connective tissue with fatty degeneration, central nuclei, and abundant degenerating fibers. Less severe myopathic features were found in muscle biopsy specimens from Patients 7 and 8.

### Methods

Biopsy specimens of quadriceps muscle were frozen in isopentane/liquid nitrogen and stored in liquid nitrogen until

use. Conventional histological and histochemical analyses were performed on 10- $\mu$ m-thick frozen transverse sections.

Dystrophin analysis was by immunohistochemistry and by immunoblot using six polyclonal antibodies (D1-2, 60 kd, 30 kd, D8, D10, and D11) obtained against six distinct fusion peptides from different portions of dystrophin, from the N-terminus to the C-terminus, as previously described [25]. Briefly, bacterial strains, kindly supplied by Dr Kunkel's group at the Children's Hospital, Boston, MA, were cultured: They contained an expression vector alone or associated with one of the six DMD gene fragments. Subsequently the bacteria were lysed; the insoluble proteins were isolated by centrifugation. The soluble proteins were purified by electrophoresis and inoculated into rabbits for production of antisera. The harvested antidystrophin antisera were diluted 1:500 to 1:600 for immunohistochemistry and 1:1,000 for immunoblot.

Immunohistochemical analysis of the DAGs adhalin and  $\beta$ -dystroglycan was performed on 6- $\mu$ m-thick cryostat sections using previously characterized antibodies [1, 3] produced in the laboratories of one of the authors (K. P. C.). Adhalin was detected with monoclonal IVD3, or sheep affinity-purified polyclonal antibodies (diluted 1:50 and 1:

100, respectively).  $\beta$ -dystroglycan was detected with rabbit or sheep affinity-purified polyclonal antibodies (diluted 1:1,000 and 1:20, respectively). Immunohistochemistry of merosin ( $\alpha$ 2 subunit in  $\alpha$ 2 $\beta$ 1 $\gamma$ 1 laminin [26]) and the laminin subunits  $\alpha$ 1,  $\beta$ 1, and  $\gamma$ 1 (previously called A, B1, and B2, respectively) was performed by using commercial monoclonal antibodies (antimerosin purchased from Chemicon, Temecula, CA, was diluted 1:800; antilaminin subunits, purchased from Gibco BRL-Life Technologies, Scotland, were diluted 1:1,000, 1:800, and 1:800, respectively).

Western blot of adhalin was performed as described [15] using a rabbit polyclonal antibody raised against synthetic peptide 44 at the dilution 1:100 [8].

Densitometric analysis of dystrophin and adhalin on immunoblots was performed as reported for dystrophin quantitation by Bulman and coauthors [27], using a densitometer and the BIO-PROFIL software system; the results were expressed as the percentage of absorbance compared to normal control values.

## Results

By immunocytochemistry dystrophin was distributed normally on the surface of all muscle fibers in all pa-

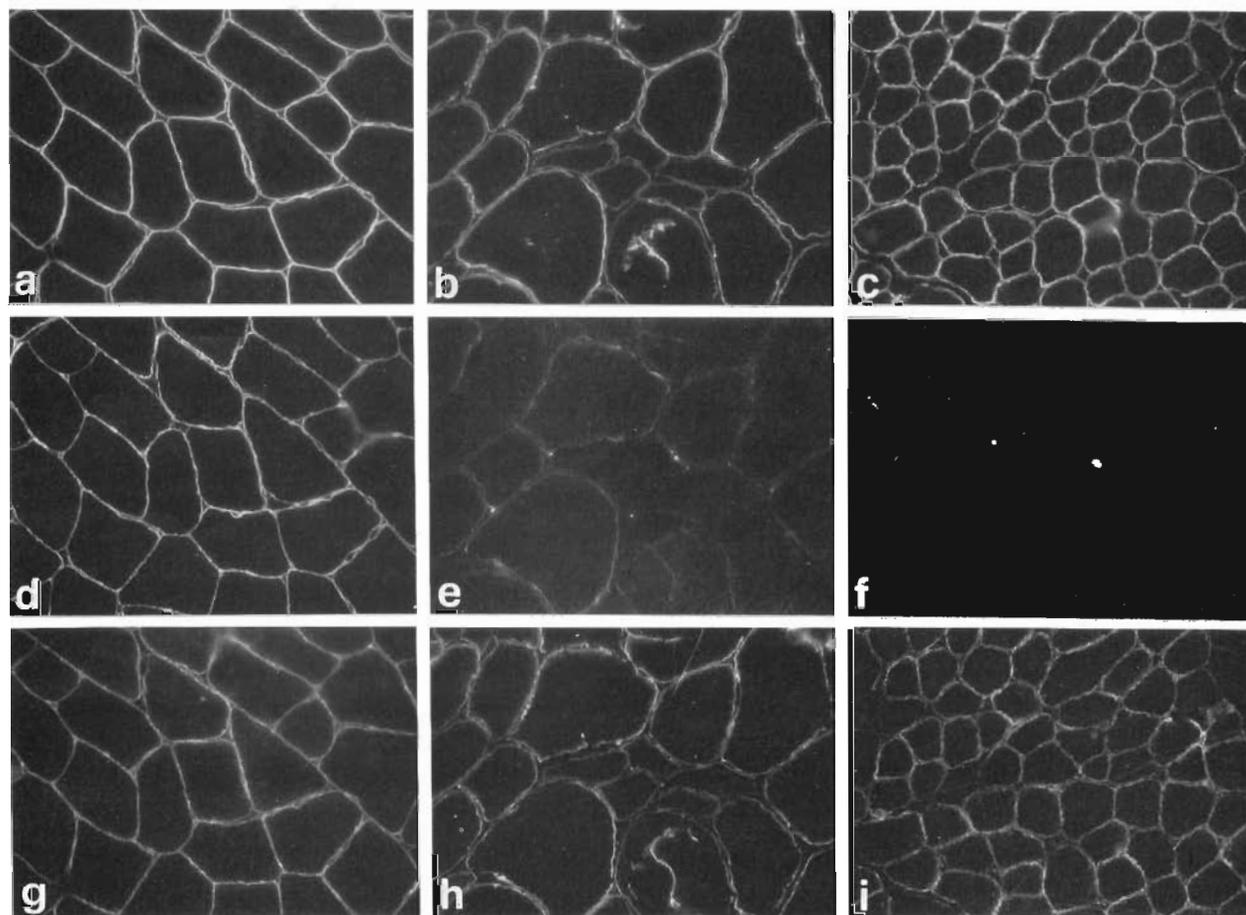


Fig 1. Dystrophin (a, b, c), adhalin (d, e, f), and  $\beta$ -dystroglycan (g, h, i) immunofluorescent staining in a normal control subject (a, d, g), Patient 2 (c, f, i), and Patient 3 (b, e, h). ( $\times 250$  before 10% reduction.)

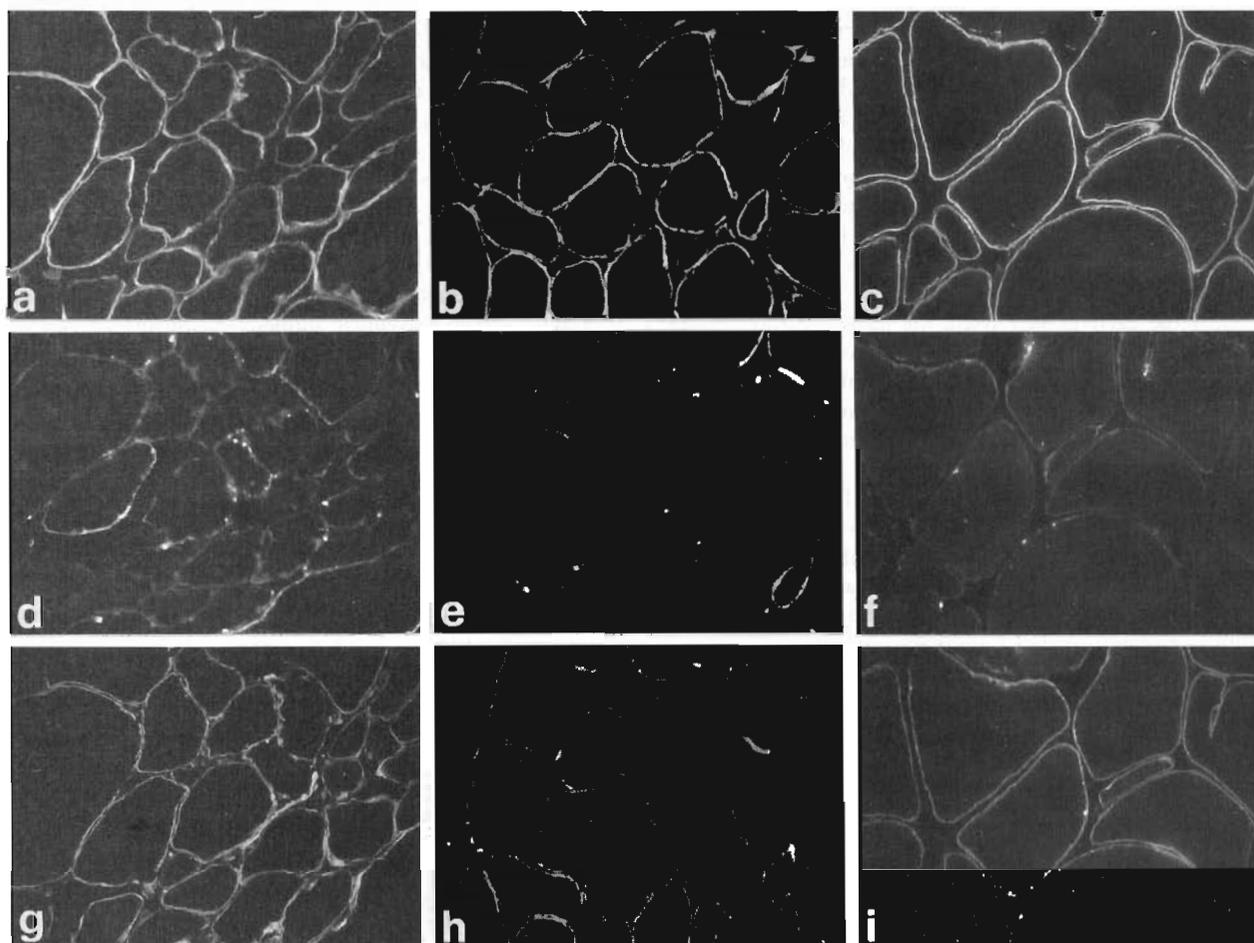


Fig 2. Dystrophin (a, b, c), adhalin (d, e, f), and  $\beta$ -dystroglycan (g, h, i) immunostaining in the 3 sisters—Patient 6 (a, d, g), Patient 7 (b, e, h), and Patient 8 (c, f, i). ( $\times 250$  before 8% reduction.)

tient specimens. A normal dystrophin band was detected by immunoblot and densitometry in all muscles examined.

In Patients 2 (Fig 1) and 5, positivity for adhalin was completely absent. In Patient 1 the intensity of adhalin immunostaining was highly reduced with a patchy distribution of the signal on most fibers; in Patients 3 (see Fig 1) and 4 adhalin expression was very faint on all fiber surfaces. Two biopsy specimens from Patient 3 were available for analysis and adhalin reduction was similar in both. In the 3 patients with familial disease (Patients 6–8), reduction of immunofluorescence intensity was variable, and unexpectedly was least reduced in Patient 6—the most severely affected of the 3 (Fig 2).

Western blot detected a band corresponding to adhalin of variably reduced intensity in Patients 1 (38.0% of normal control value), 3 (35.0%), 4 (13.8%), 6 (49.4%), 7 (42.1%), and 8 (23.0%); no adhalin band was detected in patients 2 and 5 (Fig 3).

The immunostain for  $\beta$ -dystroglycan was normal or

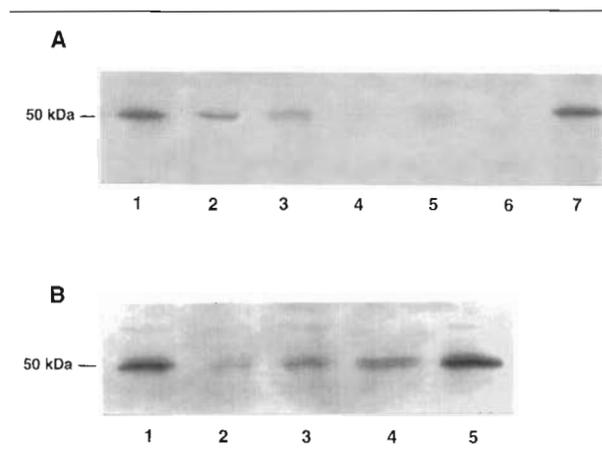


Fig 3. Adhalin immunoblots. (A) Patient 1 (lane 2), Patient 3 (lane 3), Patient 2 (lane 4), Patient 4 (lane 5), Patient 5 (lane 6), normal controls (lanes 1 and 7). (B) Patient 8 (lane 2), Patient 7 (lane 3), Patient 6 (lane 4), normal controls (lanes 1 and 5).

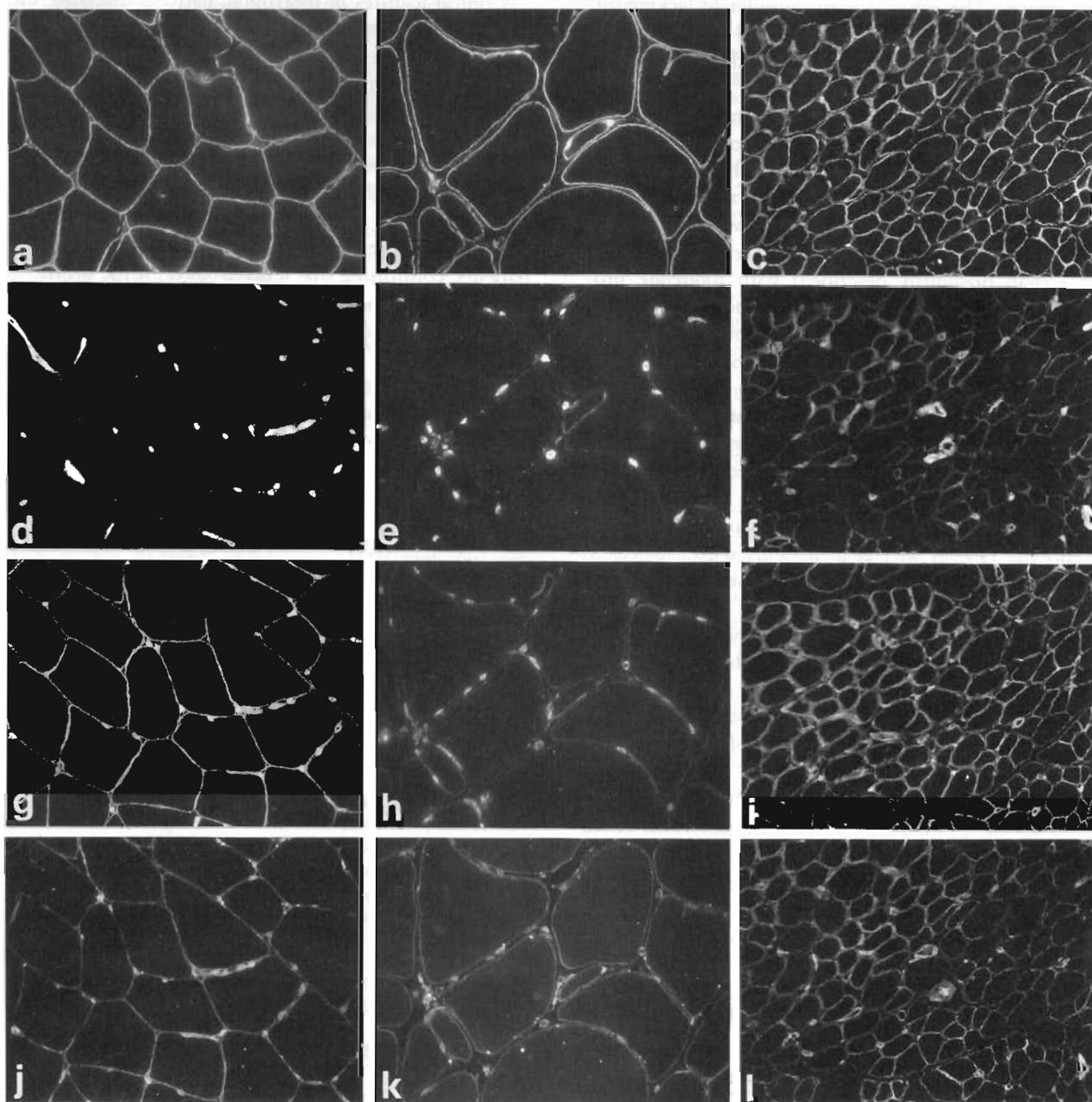


Fig 4. Immunostaining of merosin (a, b, c), laminin  $\alpha$ 1 (d, e, f), laminin  $\beta$ 1 (g, h, i), and laminin  $\gamma$ 1 (j, k, l) in a normal control subject (a, d, g, j), Patient 8 (b, e, h, k), and Patient 2 (c, f, i, l), showing reduction of laminin  $\beta$ 1 subunit in Patient 8. ( $\times 250$  before 8% reduction.)

minimally reduced in all patients. Merosin, which is expressed specifically in the basal lamina of muscle fibers, was also normally expressed, as were the laminin  $\alpha$ 1 and  $\gamma$ 1 subunits (in comparison with other patients with no adhalin deficiency and with normal control subjects). In patients 1, 4, 6, 7, and 8 the laminin  $\beta$ 1 subunit was variably reduced in the basal lamina surrounding the fibers; laminin  $\beta$ 1 was normal in Patients 2, 3, and 5 (Fig 4).

#### Discussion

In our patients, age at onset and rate of disease progression were highly variable, while the distribution of hyposthenia was similar in all and mainly involved pelvic girdle muscles. We found no apparent relation between disease severity and the quantity of adhalin expressed: 1 of the 2 patients with no adhalin expression (Patient 2) was almost asymptomatic, presenting only hypertrophic calves, but he was too young to predict disease

progression. The other patient completely lacking adhalin (Patient 5) was severely affected at age 10 and her 13-year-old sister, presumably with the same defect, was also severely affected.

Of the patients with reduced adhalin, Patient 1 had mild weakness and slow disease progression. Unfortunately a muscle sample from his cousin, who was severely affected, was not available for analysis and we cannot exclude phenotypic variability within this family. In Patients 3 and 4 the disease was late in onset (later than previously described) with moderate progression. The 3 sisters (Patients 6, 7, and 8) had similar clinical presentations, but as already noted, 1 sister was confined to a wheelchair even though she had more adhalin than her siblings.

The relationship between disease severity and adhalin expression is not, therefore, straightforward, as is also evident from previously published studies. Faint adhalin positivity was observed in patients with SCARM1 linked to the 13q12 locus and a DMD-like phenotype [15] as well as in patients with a defect in the adhalin gene located on chromosome 17 [20, 21]. In the latter condition, severity varied greatly and did not correlate closely with the quantity of protein expressed, but apparently depended on the type of mutation: Patients with homozygous null mutations were more severely affected, while patients with missense mutations and total or partial lack of adhalin had a variable clinical phenotype.

Like others [21] we found that when present, adhalin was of normal size by Western blot. This could be because an unstable protein of normal molecular weight is synthesized from a mutated adhalin gene (in primary adhalinopathy), or a normal protein is quantitatively reduced due to deficiency of another gene product associated with adhalin (in secondary adhalinopathy). In this context we note that Mizuno and colleagues [28] found a reduction of adhalin and other DGC components in SCARM1 patients.

Since the first finding of adhalin deficiency in North African families with high consanguinity, this DAG was shown to be deficient in numerous patients from various populations. Patients from families where consanguinity is absent are also known [22, 24, 29, 30]. We have now described more patients with adhalinopathy, from which it emerges that the condition is associated with considerable clinical heterogeneity, and is also likely to be relatively common among patients with muscular dystrophy. These characteristics, combined with the lack of pathognomic morphological criteria for diagnosis, lead us to suggest that immunofluorescence analysis for adhalin should become a routine part of the diagnostic workup of muscular dystrophy patients in whom dystrophin is normal. Subsequent molecular analysis would provide prognostic information as well as a basis for genetic counseling.

Clinical features of dystrophic patients directing the clinician toward possible adhalin deficiency are: proximal limb muscle weakness, more severe in the lower limbs, calf hypertrophy (often), high plasma CK levels, and dystrophic changes of muscle. Some cases of cardiomyopathy have been reported, but only in one family was heart function severely impaired [21]. Disease severity and age at onset are variable and unpredictable.

Like Higuchi and colleagues [23], we also found a reduction of the laminin  $\beta 1$  subunit, but not in all patients, confirming that a disturbance in the organization of the laminin heterotrimer (probably leading to altered sarcolemma-extracellular matrix interaction and sarcolemmal instability) is common in adhalin deficiencies. The intriguing question is the relationship between altered laminin expression and the primary adhalin defect.

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