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DEFICIENCY OF THE 50 kDa DYSTROPHIN-ASSOCIATED GLYCOPROTEIN AND ABNORMAL EXPRESSION OF UTROPHIN IN TWO SOUTH ASIAN COUSINS WITH VARIABLE EXPRESSION OF SEVERE CHILDHOOD AUTOSOMAL RECESSIVE MUSCULAR DYSTROPHY

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Abstract—Two male cousins with severe childhood, autosomal recessive, Duchenne-like, muscular dystrophy (SCARMD) have been identified with a deficiency of the 50 kDa dystrophin-associated glycoprotein but normal expression of dystrophin. Both boys were from consanguineous marriages and were Asian, having originated from Pakistan. This is in contrast to all previously reported cases from North Africa. Clinical severity varied and the patients were still able to walk at 13 and 12 yr, respectively. Neither had calf hypertrophy, a feature reported to be almost consistent in the North African patients. Abnormal expression of utrophin, the dystrophin-related protein, was observed on the surface of several non-regenerating muscle fibres, with less intense immunolabelling in the clinically more affected child. This family shows that SCARMD is not confined to North Africa and illustrates a hitherto unreported expression of utrophin in this condition.

Key words: Muscular dystrophy, autosomal recessive dystrophy, dystrophin, dystrophin-associated proteins, utrophin, dystrophin-related protein, immunocytochemistry.

INTRODUCTION

Dystrophin, the defective gene product in Duchenne muscular dystrophy (DMD) [1, 2], is associated with a complex of sarcolemmal glycoproteins [3–6] believed to link the cytoskeleton of muscle fibres with the extracellular matrix component, laminin [6]. It has been suggested that the complex has a central role in maintaining membrane stability and that deficiencies in its components may disrupt the linkage between the cytoskeleton and extracellular matrix and lead to muscle fibre necrosis [7].

Abnormalities in the expression of the dystrophin-associated glycoproteins have been found in DMD [4, 6, 8], Fukuyama congenital muscular dystrophy [9], and the severe childhood autosomal recessive form of muscular dystrophy (SCARMD), common in North Africa [10].

SCARMD is a progressive muscular dystrophy that has clinical and pathological features in common with DMD [11]. It has been mapped to chromosome 13q12 and affects males and females [12]. Age of onset and progression of the disease is variable, even within the same family, but most cases become wheelchair bound between 12 and 15 yr, and death usually results from respiratory failure in the third decade [11]. Calf hypertrophy is a common feature [11]. Morphological abnormalities in skeletal muscle are similar to those observed in other dystrophies, with variation in fibre size, fibre necrosis, regeneration and an increase in connective tissue. Dystrophin expression is normal in SCARMD [13] but the 50 kDa dystrophin-associated glycoprotein is markedly reduced, both at the sarcolemma and neuromuscular junction [10]. The 35 kDa component is also reduced in SCARMD. In contrast, the 156 and 43 kDa dystrophin-associated glycoproteins and 59 kDa dystrophin-associated protein are preserved [10].

Utrophin (dystrophin-related protein, DRP), an autosomal protein encoded by chromosome 6, with considerable homology to dystrophin [14–16], has recently been studied in several

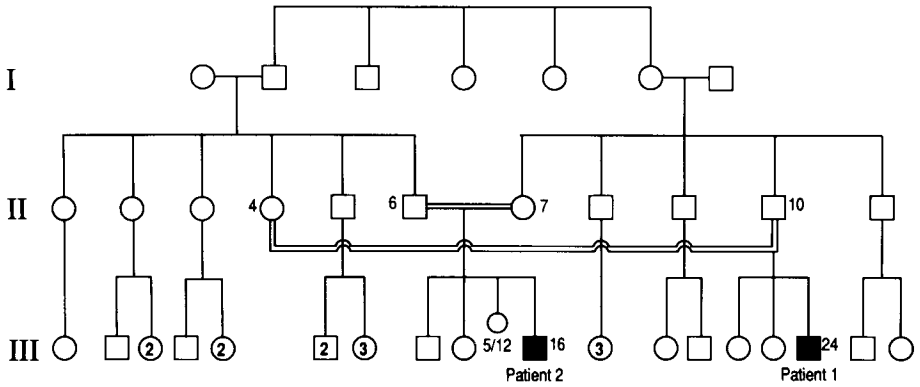

Fig. 1. Family tree of the two patients studied (index cases III, 24 and III, 16). Note the consanguinity of both pairs of parents (II, 4 & 10 and II, 6 & 7, respectively).

neuromuscular disorders [17–20]. The 13 kb transcript has a molecular mass of approximately 400 kDa, slightly smaller than dystrophin, but, unlike dystrophin, it is expressed in several visceral tissues in addition to muscle and neural tissues [21, 22]. In normal adult muscle, utrophin is confined to the neuromuscular and myotendinous junctions and blood vessels [17–20], and is absent from the extrajunctional sarcolemma. It is, however, expressed on the surface membrane of regenerating fibres in a variety of neuromuscular disorders [19], on human fetal fibres until about 26 weeks of gestation [23], and on several mature fibres in Duchenne and Becker dystrophies (DMD, BMD) and inflammatory myopathies [17–20]. Utrophin expression has been reported to be normal in other neuromuscular disorders [19, 20], including three cases of SCARMD [17].

We report here two cousins of Asian origin with a deficiency of the 50 kDa dystrophin-associated glycoprotein, in whom we have also examined the immunocytochemical localization of utrophin.

PATIENTS AND METHODS

The two patients were male first cousins from consanguineous marriages. The family tree (Fig. 1) shows that all four parents were first cousins and that a brother and sister in one family married a brother and sister in the other. The four parents had common grandparents and are clinically normal. Both patients were born in Britain but the parents are all of Asian origin. The father of patient 1 (II, 10) and the mother of patient 2 (II, 7), brother and sister, are Kenyan Asians, whilst the mother of patient 1 (II, 4) and the father of patient 2 (II, 6), sister and brother, are from Pakistan. No links to North Africa have been traced. No abnormalities in dystrophin DNA have been found in either patient, and the family tree excludes an X-linked mode of inheritance, in view of the male to male transmission in family 2.

Patient 1 (Pedigree III, 24)

This patient was born after a normal pregnancy at 36 weeks gestation. Early milestones were delayed and he did not sit unsupported until about 1 yr of age, and walked at 18 months. His parents were unconcerned until $2\frac{1}{2}$ yr of age when he was noted to walk on his toes and to have a waddling gait. During childhood he was generally weak and often fell. His intellectual development was normal.

He was referred to the Neuromuscular Unit at Hammersmith Hospital at the age of 9 yr when he was noted to be small and thin, his height (121 cm) and weight (15.4 kg) being below the 3rd centile. He had a waddling gait and could not jump or hop. He got up from the floor with a Gowers manoeuvre in 5 s and had a motor ability score of 30 out of 40 [24]. There was no scoliosis or calf hypertrophy, and no cardiac abnormalities. Serum creatine kinase (CK) was 9925 IU l^{-1} (normal < 170 IU l^{-1}). When last assessed at 12 yr of age, his weakness was relatively stable. He was still able to walk 150 ft in 55 s but his motor ability score was down to 27 and the Gowers manoeuvre took 20 s. He had no scoliosis.

Patient 2 (Pedigree III, 16)

This male patient was born at term after a normal pregnancy. His motor milestones were normal and he sat at 5 months, stood at 10

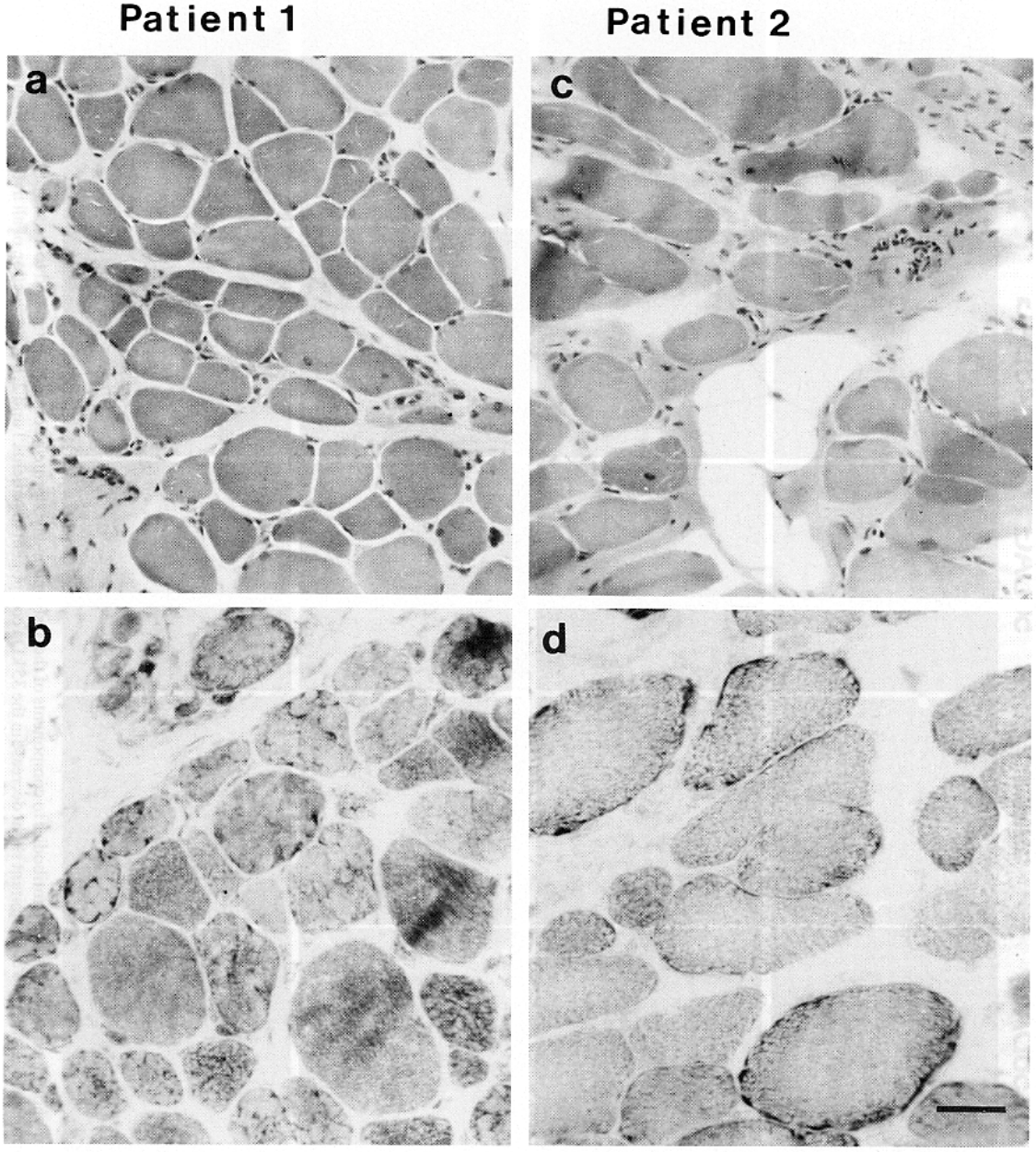

Fig. 2. Muscle biopsies of patient 1 (a, b) and patient 2 (c, d) stained with haematoxylin and eosin (a, c) showing features of dystrophic muscle, and NADH tetrazolium reductase (b, d) showing two fibre types and abnormal deposits in the small fibres in patient 1. Bar = 50 μm.

months and walked at 12 months. His development caused no concern until about 8 yr of age, when he was noted to have difficulty running. He was referred to us aged 10 yr. His height (131 cm) was at the 10th centile, his weight (23.7 kg) below the 3rd. He had a waddling gait, difficulty climbing stairs, a Gowers manoeuvre of 4 s and a motor ability score of 38. He could jump and also hop on one leg, but with difficulty. He had no calf hypertrophy and no cardiac abnormalities. His serum CK was 7360 IU l^{-1}. He was notably less affected than his cousin.

Muscle biopsy

Needle muscle biopsies [25] were obtained from the quadriceps of the patients at 9 and 10 yr of age, respectively. Samples were mounted on cork, transversely orientated, and rapidly frozen in isopentane cooled in liquid nitrogen. Cryostat sections (10 μm) were stained histologically and histochemically according to established methods [26] and serial sections (6 μm) were immunostained with antibodies to dystrophin, dystrophin-associated proteins (DAP), β-spectrin, utrophin and myosin heavy chain isoforms.

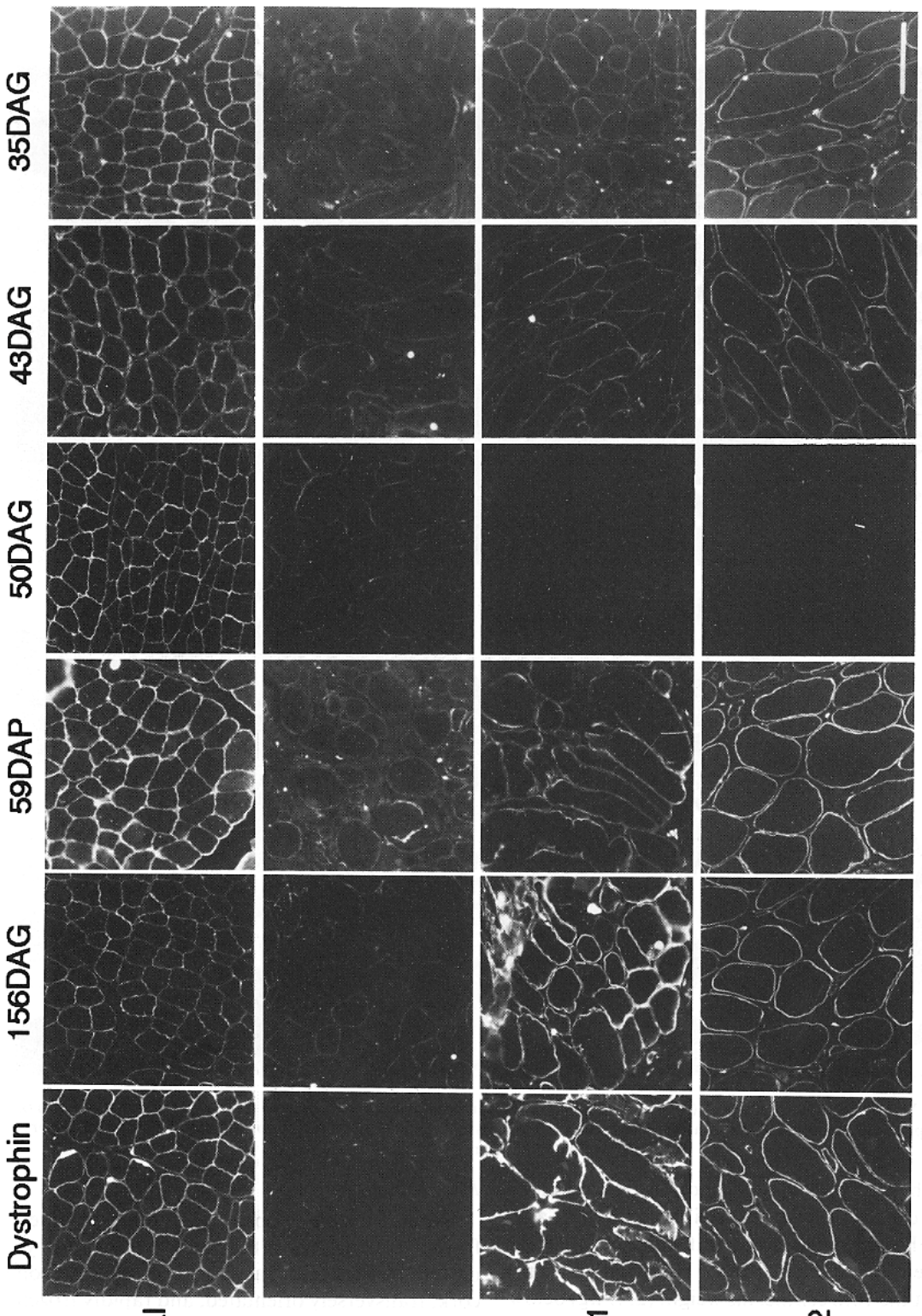

Fig. 3. Muscle biopsies from both patients immunostained with a panel of antibodies to components of the dystrophin–glycoprotein complex, compared to a control case and a patient with DMD. Note the absence of the 50 kDa glycoprotein (50DAG) and concomitant slight decrease in the 35 kDa component in patients 1 and 2, but a reduction of all five dystrophin-associated proteins in DMD muscle. Bar = 100 μm.

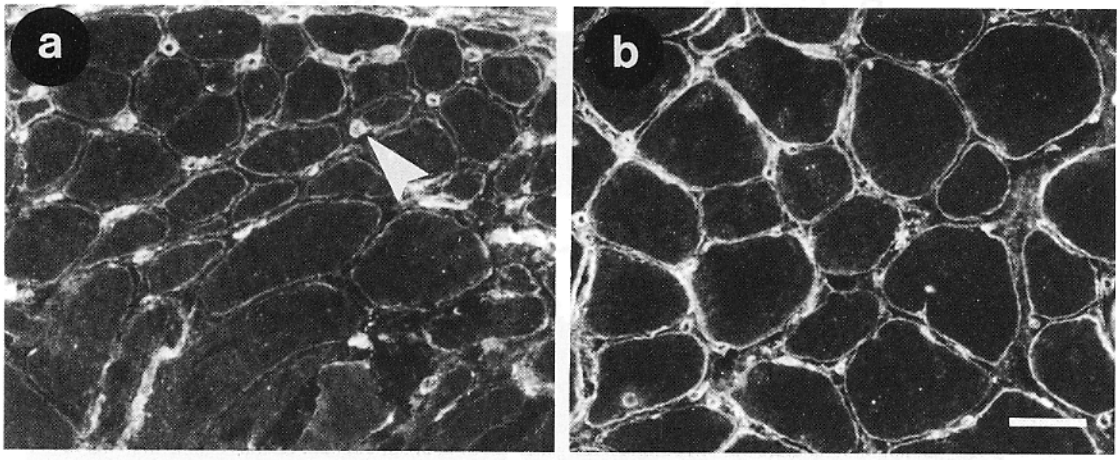

Fig. 4. Immunolabelling of utrophin in the biopsies of patient 1 (a) and patient 2 (b) showing sarcolemmal expression of utrophin on most fibres in both cases, but less intense fluorescence in patient 1 (labelled in parallel, same photographic exposure). Capillaries are also labelled with antibodies to utrophin (arrow). Bar = 50 μm.

Antibodies and immunocytochemistry

All antibodies have been characterized previously. A mouse monoclonal antibody (VIA4[2]) to the C-terminal domain of dystrophin was used neat [4, 9, 10], and a rabbit polyclonal antibody (P6) corresponding to the C-terminus of the rod domain of dystrophin (amino acids 2814–3028) was diluted 1:1000 [27]. Antibodies to the 156, 59, 50, 43 and 35 kDa components of the dystrophin-associated complex were raised in sheep and used neat (156 K), or at dilutions of 1:20 (59 K, 50 K, 43 K and 35 K) [6, 8, 9, 10, 28]. A monoclonal antibody to the 50 kDa component ($IVD3_1$) was diluted 1:50 [8, 10]. Sections (6 μm) were incubated for 2 h with the primary antibody. For the sheep antibodies this was followed by the appropriate biotinylated secondary antibody (1:500) for 1 h, and fluorescein-conjugated streptavidin (1:1000) for 30 min. Following the monoclonal antibodies sections were labelled with fluorescein-conjugated anti-mouse IgG–IgM (1:200) for 1 h. The P6 antibody to dystrophin was incubated for 30 min and detected with streptavidin conjugated to Texas Red (1:200) for 15 min. Serial sections from the two patients were processed with sections of normal muscle and of DMD muscle on the same slide. β-Spectrin was localized with antibody 56A (diluted 1:1) [23] and utrophin with the specific monoclonal antibody MANCHO 7 [21], diluted 1:5. Both were labelled for 30 min with the primary antibody and visualized with a biotinylated secondary antibody (1:200, 30 min) followed by streptavidin–Texas Red (1:200, 30 min). Fetal, fast and slow myosin heavy chain isoforms were localized with previously characterized monoclonal antibodies [29, 30], using a similar Texas Red fluorescence technique. All washings and dilutions were in phosphate buffered saline. Control sections were incubated without primary antibodies and showed only autofluorescence.

RESULTS

In both cases the muscle sections showed a dystrophic picture with an abnormal variation in fibre size, split fibres, internal nuclei and an increase in fat and connective tissue (Fig. 2a, c). There was a two-fibre pattern with ATPase staining, with a slight predominance of type 1 fibres in patient 2. In patient 1 oxidative enzyme staining with the NADH tetrazolium reductase technique revealed a population of fibres with prominent peripheral deposits of stain. These resembled lobulated fibres but the peripheral areas were less marked than those usually seen in lobulated fibres (Fig. 2b). Patient 2 also showed peripheral deposits stained with NADH tetrazolium reductase in some larger fibres, but these were more linear than those in patient 1 (Fig. 2d).

Immunolabelling for dystrophin and β-spectrin was normal. Both patients, however, showed an absence of the 50 kDa dystrophin-associated glycoprotein (Fig. 3). Some reduction in the 35 kDa component was also noted, in comparison with normal muscle, but this was less marked than in DMD muscle (Fig. 3).

Utrophin was detected on several fibres in both patients, but the intensity was variable. In patient 1 several fibres expressed utrophin, but the intensity of fluorescence was weaker

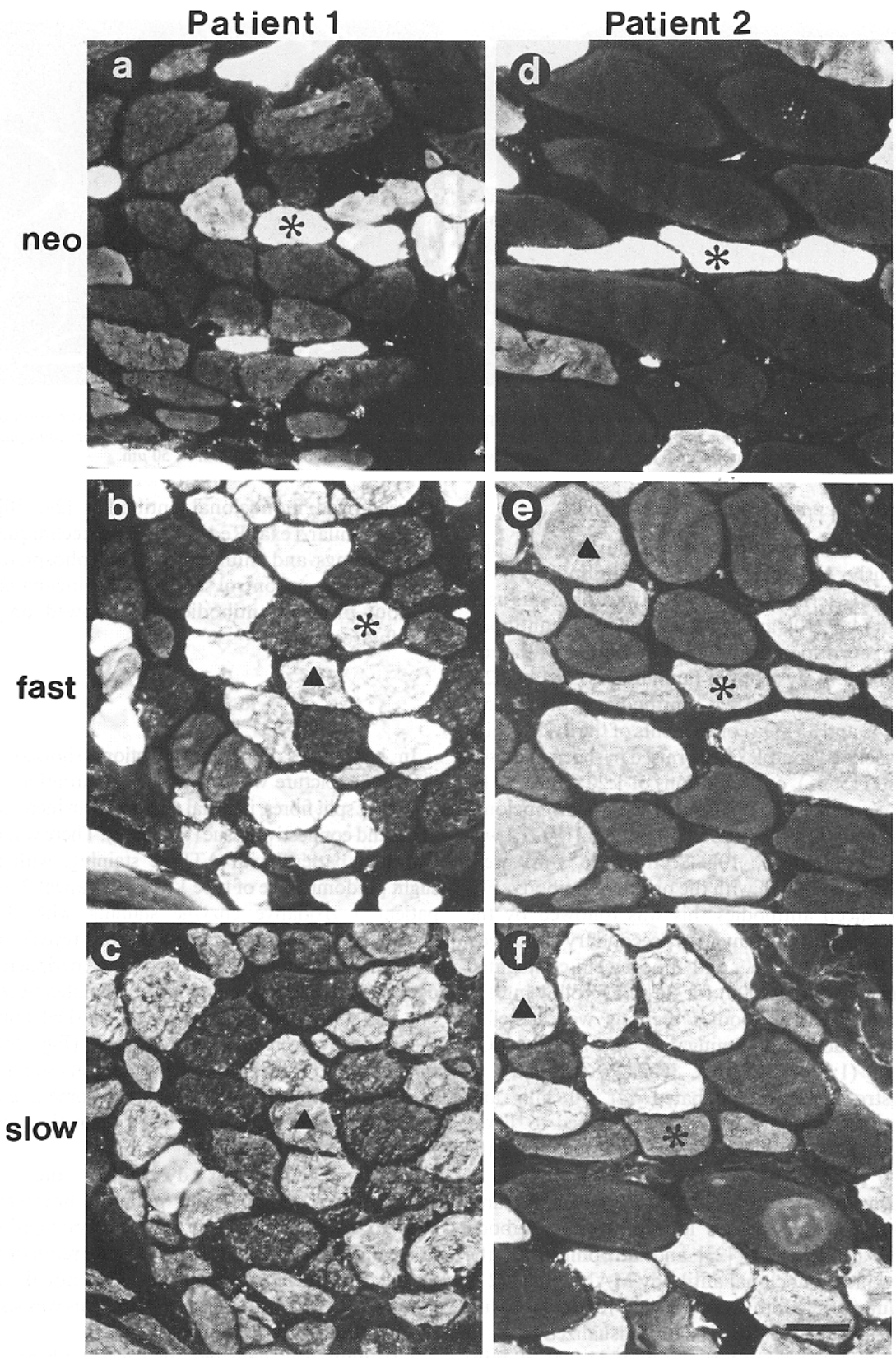

Fig. 5. Immunolabelling of fetal (= neo, a, d), fast (b, e) and slow (c, f) myosin heavy chain isoforms in biopsies from patient 1 and 2, showing fetal myosin in several fibres and co-expression of more than one isoform in some fibres (* and ▲), and a slight predominance of fibres with slow myosin in patient 2. Bar = 50 μm.

compared with that in patient 2, stained in parallel (Fig. 4). Capillaries and blood vessels expressed utrophin and it was also detected in split fibres. Utrophin was co-expressed with fetal myosin in some, but not all, fibres.

There was a slight predominance of fibres expressing slow myosin in patient 2, although the intensity of fluorescence was variable. Co-expression of slow myosin with fast and/or fetal myosin was seen in both patients (Fig. 5).

DISCUSSION

Using specific antibodies to the dystrophin-associated proteins we have identified two male cousins who lack the 50 kDa component. Our patients, therefore, show a similar defect to cases of severe childhood autosomal recessive muscular dystrophy (SCARMD) described previously [10]. This autosomal form of muscular dystrophy is prevalent in North Africa, but our Asian cases, originating from the Indian subcontinent, show that it is likely to be a more widespread disorder. Recent studies of European families have also identified cases deficient in the 50 kDa glycoprotein (F. M. S. Tomé, personal communication), and it is probable that other cases will be identified when antibodies to the glycoproteins are more widely used.

Linkage analysis has shown that the defect in SCARMD is located on chromosome 13q12 [12] but it is not yet known if the 50 kDa glycoprotein is coded by a gene in this region, or if its absence is a secondary phenomenon. Linkage analysis in our family is in progress. The previous report of the dystrophin-associated proteins in patients with SCARMD noted a reduction in immunolabelling of the 35 kDa component, in addition to the absence of the 50 kDa glycoprotein [10]. Our cases confirm this observation. In contrast to DMD, there is no significant reduction in the 156 and 43 kDa glycoproteins or the 59 kDa protein.

The clinical features of SCARMD resemble those in DMD, both in their mode of onset and progression [11–13]. Calf hypertrophy has been reported to be an almost consistent feature of SCARMD [11] but neither of our cases showed this feature. Clinical variability between cases, even in members of the same family, has also been reported [11] and it is interesting to note that of the cases presented here patient 1 was more severely affected, and had an earlier onset of symptoms, than patient 2. Loss of ambulation in SCARMD is more variable than in DMD and can occur from 10 to 20 yr. DMD patients, in contrast, are wheelchair bound by 12 yr. Although case 1 is more severely affected than his cousin, he seems to be at the milder end of the clinical spectrum and is currently still ambulant at 13 yr of age. His cousin, patient 2, is considerably milder and his parents were unconcerned until the boy was about 8 yr of age, believing that he had a different problem to that of his cousin. In addition, neither of our patients had cardiac involvement, a feature which is characteristic of Duchenne and Becker muscular dystrophy.

The cases presented are also of interest in relation to the expression of utrophin in the muscle fibres. Hitherto, extrajunctional sarcolemmal expression of utrophin in human muscle has been confined to immature and regenerating fibres, and mature fibres in DMD, BMD and inflammatory myopathies. Thus, SCARMD and DMD also show similarities in an abnormal expression of utrophin. In SCARMD this is accompanied by a normal expression of dystrophin, in contrast to its absence, or considerable reduction, in most cases of DMD. Utrophin was also detected in split fibres, in common with other membrane associated proteins and extracellular matrix proteins [32]. The origin of the splits is uncertain, and the transverse sections used in our cases give only a limited anatomical view. The association of utrophin with immaturity, however, raises the possibility that they could either result from incomplete fusion after regeneration, or be new membranes that are going through a developmental transition.

Although some fibres expressed the fetal isoform of myosin, probably in relation to regeneration and immaturity, several fibres labelled with antibodies to utrophin did not express fetal myosin, showing that they were mature fibres. During regeneration of normal dog muscle (manuscript in preparation) and during development of human fetal muscle fibres [23] we have shown that utrophin is down-regulated before fetal myosin. Thus, the presence of utrophin in dystrophic muscle fibres, in the absence of fetal myosin, is unlikely to relate directly to regeneration. The reasons for the abnormal utrophin expression, however, are not known. It has been suggested that utrophin might compensate for the loss of dystrophin in DMD muscle and might offer some protection against necrosis [20]. In this context it is interesting to note that in the less severely

affected SCARMD case, (patient 2) we detected more utrophin immunocytochemically. Caution is needed, however, in the absence of quantitative data on the relative amounts of utrophin, and in interpreting intensities of immunofluorescence. Quantitation of utrophin by Western blot is difficult because of the contribution of the vascular tissue and regenerating fibres, both of which vary from sample to sample. The number of capillaries varies in relation to the number of type 1 fibres and, as the proportion of these is frequently increased in diseased muscle, quantitation of utrophin must take account of several factors, and be carefully controlled, before meaningful data can be obtained.

It is also of interest to note that patient 2, who showed greater expression of utrophin, had normal early motor milestones. It would be of interest to know if utrophin is already detected at early stages in these milder cases, before clinical signs are apparent, and to compare them with more severely affected cases.

Myosin isoform expression in our two cases of SCARMD resembles that seen in other autosomal dystrophies, such as limb girdle dystrophy, and in DMD and BMD [31, 32]. The presence of fetal myosin is probably the result of regeneration, and in most species it takes about 3 weeks to be down-regulated [33]. Fetal myosin expression is, therefore, seen in histologically normal fibres, as well as in small basophilic fibres. Studies of regeneration of normal and dystrophic muscle also suggest that co-expression of fast and slow myosin may, at least in part, result from regeneration [32].

In summary, we have identified two South Asian male cousins with a variable clinical phenotype of severe childhood autosomal Duchenne-like muscular dystrophy who have a deficiency of the 50 kDa dystrophin-associated glycoprotein and an associated abnormal sarcolemmal expression of utrophin.

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