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Monoclonal antibodies detect a spectrin-like protein in normal and dystrophic human skeletal muscle

(Duchenne muscular dystrophy/immunocytochemistry/cytoskeleton/plasma membrane)

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ABSTRACT **Spectrin is the major protein of the erythrocyte membrane skeleton, which is bound to the cytoplasmic surface of the membrane's lipid bilayer and is responsible for cell shape and membrane elasticity. Inability to identify spectrin in other cell types led to the assumption that this protein was unique to erythrocytes. However, spectrin-like proteins have been demonstrated recently in a variety of cell types, including skeletal and cardiac muscle, in several species. We used monoclonal antibodies against human erythrocyte spectrin subunits in an immunocytochemical study to detect related proteins in normal and diseased human skeletal muscle. Six of seven monoclonal antibodies against β-spectrin determinants were bound at the cytoplasmic surface of muscle fiber plasma membranes, whereas none of six monoclonal antibodies against α-spectrin determinants was bound. Muscle fibers of patients with neuromuscular diseases showed similar distribution and specificity of antibody binding to those of normal subjects, but the intensity of binding was increased. In contrast, probable regenerating fibers in muscle of patients with muscular dystrophies showed reduced binding of antibodies, but reduced binding was not seen in fetal muscle fibers nor in those of a patient with a myotubular myopathy. We conclude that human skeletal muscle fibers possess a spectrin-related protein associated with their plasma membrane that shows extensive β-chain similarities to erythrocyte spectrin but differs significantly with respect to the α-subunit. Its function may be associated with the maintenance of membrane and myofibril integrity during contraction, and the increased antibody binding in diseased muscle may reflect a structural rearrangement of spectrin or a compensatory increase in spectrin abundance in response to increased stress on these systems.**

Plasma membrane-associated proteins immunologically related to, and sharing biochemical properties with, erythrocyte spectrin have been described recently in a variety of cell types (1–10), and these findings have been reviewed (11, 12). Previously, spectrin, the major component of the erythrocyte membrane skeleton, was believed to be unique to erythrocytes (13, 14).

Native erythrocyte spectrin is a 200-nm-long tetramer consisting of two heterodimers, each of one α subunit (band 1; M_r 240,000) and one β subunit (band 2; M_r 220,000) (15). Spectrin is crosslinked by actin (band 5) and band 4.1 to form a protein meshwork (16), the membrane skeleton, which is laminated to the cytoplasmic surface of the plasma membrane's lipid bilayer by indirect interaction of spectrin with band 3 (the integral anion channel protein) through ankyrin (band 2.1) (17, 18). This meshwork is believed to confer upon the erythrocyte membrane its great strength and elastic deformability. The description of spectrin-like proteins (hereafter referred to as "spectrins") in cells other than erythrocytes now raises the possibility of a similar linkage in these cells, with implications for the maintenance of membrane integrity, cell shape, membrane–cytoskeleton interactions, and movement.

Nonerythroid spectrins that have been isolated and characterized are also composed of two dissimilar polypeptide chains, with molecular masses close to those of their erythrocyte counterparts. The best characterized exmples, porcine brain spectrin (fodrin) and terminal web protein of chicken intestinal brush borders (TW 260/240) have been shown by electron microscopy to resemble erythrocyte spectrin in size and shape, and it has been suggested on this basis that they are $\alpha_2\beta_2$ tetramers (3, 19). In addition, brain spectrin has been shown to bind to erythrocyte membranes stripped of spectrin, to ankyrin, to F-actin (which it gels), and to calmodulin in a manner similar to that of erythrocyte spectrin (3–5, 7, 19, 20).

Immunocytochemical studies with polyclonal antisera raised against erythrocyte or brain spectrin have shown that spectrin-related proteins are present at the plasmalemma of a wide variety of cell types, including chicken (6, 8) and guinea pig (2) skeletal muscle. However, of these, only the brain and terminal web proteins have been isolated so far.

We have described, in a preliminary communication, the immunocytochemical localization of such a protein in normal and dystrophic human skeletal muscle, using monoclonal antibodies to erythrocyte spectrin subunits (21). An erythrocyte-type membrane skeleton would have particular functional significance in skeletal muscle fibers, especially regarding the implied role of structural membrane defects in the etiology of muscle disease and, in particular, of Duchenne muscular dystrophy (DMD) (22, 23), an X-chromosome linked disease characterized by progressive muscle wasting, leading to death in the second or third decade of life. We have investigated the crossreactivity of 13 distinct anti-spectrin monoclonal antibodies with human skeletal muscle proteins in frozen tissue sections and also have compared the distribution of crossreacting proteins in normal muscle with that of patients with various neuromuscular diseases.

MATERIALS AND METHODS

Muscle Samples. Needle biopsy samples were obtained from the quadriceps muscle of five patients with DMD (age, 2–9 yr) and from one genetically proven and two possible carriers of DMD (age, 16–36 yr). Control biopsies were obtained from four normal volunteer subjects (age, 27–39 yr) and from two boys with suspected metabolic myopathies but apparently normal biopsies (age, 10 and 19 yr). In order to assess the specificity of any abnormality found, muscle biop-

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Abbreviation: DMD, Duchenne muscular dystrophy.

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sy samples were examined also from patients with Becker muscular dystrophy (two), limb girdle muscular dystrophy, spinal muscular atrophy (two), myotubular myopathy, and peripheral neuropathy. Two samples of fetal muscle were also examined.

The tissue was frozen in liquid Arcton 12 (CCl_2F_2), cooled to near its freezing point in liquid nitrogen, and 10-μm cryostat sections were cut and stored on glass coverslips at −40°C.

Monoclonal Antibodies. Spectrin obtained by low-ionic-strength extraction from fresh human erythrocyte ghosts and purified by gel filtration on Sepharose CL-4B (Pharmacia) (15) was used to immunize BALB/c mice. Monoclonal antibodies were raised by conventional techniques, and their specificities against spectrin subunits were determined by binding to nitrocellulose filters onto which the separated spectrin polypeptides had been transferred after fractionation in $NaDodSO_4$/polyacrylamide slab gels (24). For immunocytochemical labeling, ascites fluids obtained by peritoneal drainage of Pristane-primed mice, previously injected intraperitoneally with hybridoma cells, were used either directly or, in the case of monoclonal 718/a, after fractionation by cold 40% ammonium sulfate precipitation followed by chromatography on DE-52 cellulose (Whatman).

Immunocytochemical Staining. Proteins sharing antigenic determinants with human erythrocyte spectrin were localized by indirect immunocytochemistry by sequential incubations of unfixed sections at 22°C with the appropriate mouse anti-spectrin monoclonal antibody (diluted 1:100 or 1:1,000), affinity-purified biotinylated goat anti-mouse IgG antibody (diluted 1:50; TAGO, Tissue Culture Services, Slough, U.K.), and an avidin–biotin–peroxidase complex (25) prepared according to the manufacturer's instructions (Vectastain, Sera-Lab, Crawley Down, Sussex, U.K.). Sites of antibody binding were visualized by incubation in a solution of 0.03% diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 for 5 min at 22°C, resulting in an insoluble brown reaction product. All reagents were prepared in phosphate-buffered saline. Sections were counterstained with hematoxylin and then dehydrated, cleared, and mounted. For antisera that apparently failed to crossreact with muscle, the procedure was repeated with biotinylated goat anti-mouse IgM antibody to confirm that the primary antibody was not of that type. Sections were photographed with a Zeiss photomicroscope and Ilford Pan F film.

For electron microscopy, 30-μm cryostat sections were obtained on Thermanox plastic (Imperial Chemical Industries) and were treated as above, except that after incubation they were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 30 min at 22°C, followed by 1% osmium tetroxide in the same buffer for 30 min at 22°C. They then were dehydrated and embedded in Epon. Gold ultrathin sections were cut and examined unstained or stained with uranyl acetate on an AEI Corinth 275 electron microscope.

RESULTS

Thirteen IgG anti-human erythrocyte spectrin monoclonal antibodies were used to test for the presence of spectrin-related polypeptides in frozen sections of human skeletal muscle. Six of these (39A, 52A, 56A, 58A, 63B, and 65A), all specific for the β subunit of spectrin, bound uniformly and consistently to the cytoplasmic surface of the plasma membrane of all fibers in normal human quadriceps muscle samples and to those of boys with suspected metabolic myopathies, there being no difference in binding discernable between fiber types. Monoclonal antibodies 39A, 56A, 58A, and 65A exhibited a high affinity for the membrane, giving a strong reaction when used at 1:1,000 dilution (Fig. 1*a*), whereas antibodies 52A and 63B showed a lower affinity as judged from the weaker intensity of brown reaction product, even when used at 1:100 dilution (Fig. 1*b*). The other seven antibodies tested [718/a, 61A, 66B, 88A, 93B, 95A (specific for the α subunit), and 72A (specific for the β subunit of spectrin)] showed no crossreaction, even when used at 1:100 dilution (Fig. 1*c*) or lower. In control incubations, the omission of primary and secondary antibodies showed there to be no nonspecific binding of the developing goat anti-mouse IgG and no endogenous peroxidase activity within the muscle fibers, so that the sections resembled those shown in Fig. 1*c* and *f*.

Diseased muscle fibers, with a few exceptions described below, showed a similar distribution and specificity of crossreactivity with anti-spectrin monoclonal antibodies. Antibodies 718/a, 61A, 66B, 72A, 88A, 93B, and 95A again failed to bind (Fig. 1*f*), but antibodies 39A, 52A, 56A, 58A, 63B, and 65A were all bound at the plasma membrane of the majority of fibers, giving a reaction product which, for some of the antibodies, was more intense than that obtained in normal biopsies (Fig. 1 *d* and *e* and Fig. 2 *c* and *e–h*; compare with Fig. 1 *a* and *b*).

Staining was particularly strong beneath satellite cells, the membranes of which were themselves unreactive (Fig. 2*d*). In DMD and Becker muscular dystrophy biopsies, some small fibers with prominent internal nuclei, which are presumed to be regenerating fibers, showed only weak crossreactivity (Fig. 2*a*), and some necrotic fibers bound no antibodies (not shown). In the case of the latter, it is probable that their plasma membrane was severely damaged or lost (26). In split and whorled fibers, which are commonly present in diseased muscle, the membrane that constituted the splits and whorls bound the antibodies to the same degree as did the plasma membrane (Fig. 1 *d* and *e* and Fig. 2 *f* and *g*). With this exception, no binding to internal membranes or cytoplasmic components was observed in normal or in diseased muscle fibers.

Muscle fibers of definite and possible DMD carriers showed a normal pattern of crossreactivity with anti-spectrin monoclonal antibodies (not shown). Fetal muscle fibers and those of the patient with myotubular myopathy also resembled normal adult fibers in the distribution and specific antigenicity of their crossreacting protein (Fig. 2 *b* and *c*).

When immunostained cryostat sections were embedded, sectioned, and examined in the electron microscope, it was confirmed that the reaction product was localized adjacent to the cytoplasmic surface of the plasma membrane and was not associated with any other membrane or organelle (Fig. 3).

DISCUSSION

From our results it is clear that human skeletal muscle fibers contain a protein that shares some, but not all, of the antigenic determinants of human erythrocyte spectrin. This protein is localized exclusively on the cytoplasmic surface of the plasma membrane. These observations are in general agreement with immunofluorescence studies of normal chicken (6, 8) and guinea pig (2) skeletal muscle, which used polyclonal antisera to chicken erythrocyte α and β spectrin and to guinea pig fodrin, respectively.

However, by using monoclonal antibodies we have shown that there are significant differences between human skeletal muscle and erythrocyte spectrins. This is particularly true of erythrocyte α-spectrin antigenic determinants because none of the six antibodies specific for α spectrin that we have tested crossreacted with muscle spectrin. Conversely, erythrocyte β-spectrin determinants would seem to be highly conserved between the two tissues because six of seven antibodies specific for this subunit did crossreact with muscle spectrin. These results contrast with observations (19) that erythrocyte, terminal web, and brain spectrins of chicken share a closely related and perhaps identical calmodulin

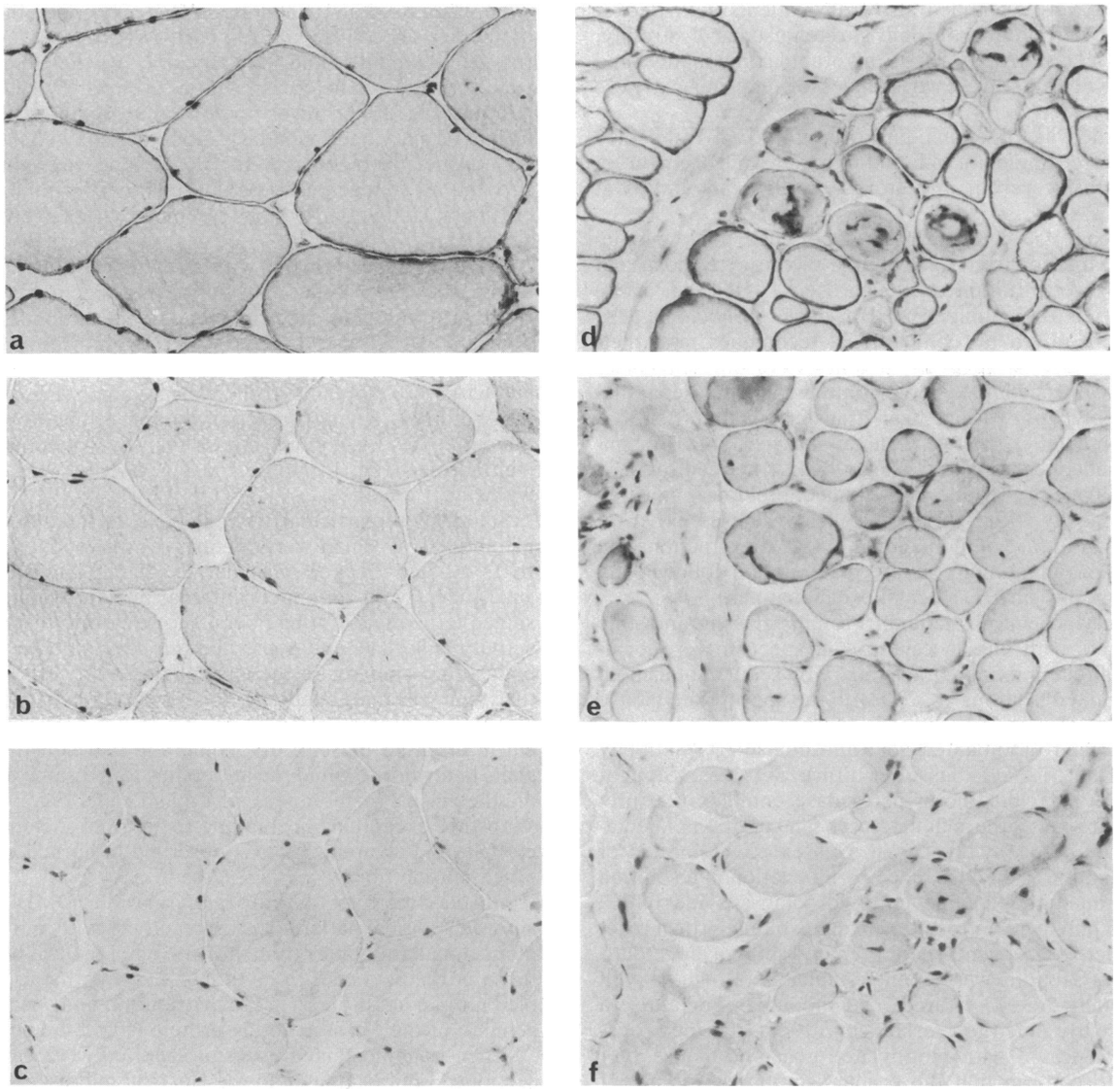

FIG. 1. Binding of monoclonal anti-spectrin antibodies to quadriceps muscle fibers of normal subjects (*a–c*) and patients with DMD (*d–f*). (Anti-α-spectrin ascitic fluids 61A and 95A, were diluted 1:100; anti-β-spectrin ascitic fluid 56A was diluted 1:1,000, and 52A was diluted 1:100.) (*a*) Normal, 56A. (*b*) Normal, 52A. (*c*) Normal, 95A. (*d*) DMD, 56A; note whorled fibers. (*e*) DMD, 52A; note split fiber. (*f*) DMD, 61A; note whorled and split fibers. Sections were counterstained with hematoxylin. (×220).

binding subunit, equivalent to erythrocyte α spectrin, but each has a tissue-specific, morphologically and antigenically distinct, second subunit. Our findings give support to the suggestion that human erythrocyte α spectrin may be distinct from nonerythroid spectrins and divergent from avian erythrocyte spectrin (11). Chicken skeletal muscle spectrin has been shown to crossreact with polyclonal antisera to chicken erythrocyte α- and β-spectrin subunits (8). In contrast, we have shown that human muscle spectrin crossreacts with monoclonal antibodies to the β but not the α subunit of human erythrocyte spectrin. Clearly the subunit composition of human muscle spectrin and the extent of its structural homology with erythrocyte spectrin requires further investigation. Monoclonal antibodies will be of great value in assessing the latter.

We found no consistent qualitative difference in the distribution of spectrin in normal and diseased muscle fibers nor in the specificity of its crossreaction with the different monoclonal antibodies. However, some histologically abnormal fibers exhibited an abnormal distribution of spectrin. Our observation that spectrin is present on the membranes that constitute splits and whorls in diseased fibers suggests that these are of plasma membrane type, in agreement with previous observations that collagen is present within such structures (27). This was the only intermyofibrillar localization of crossreactive protein that was encountered in the present study. It has been reported that in addition to the normal plasma membrane localization, dystrophic chicken skeletal muscle fibers bound polyclonal antibodies to chicken erythrocyte α spectrin throughout the sarcoplasm, primarily in punctate and needle-like aggregations (28). We have not found any such binding in dystrophic human muscle fibers using our monoclonal antibodies.

The low degree of crossreactivity in putative regenerating fibers in dystrophic biopsies prompted us to examine fetal muscle fibers and those of a patient with a myotubular myopathy. However, such fibers were found to have strong binding of antibodies, similar to that of normal adult fibers; therefore, the significance of the apparently small amount of spectrin in regenerating fibers remains obscure.

Finally, there was evidence of an increased binding of anti-β-spectrin monoclonal antibodies in diseased muscle fi-

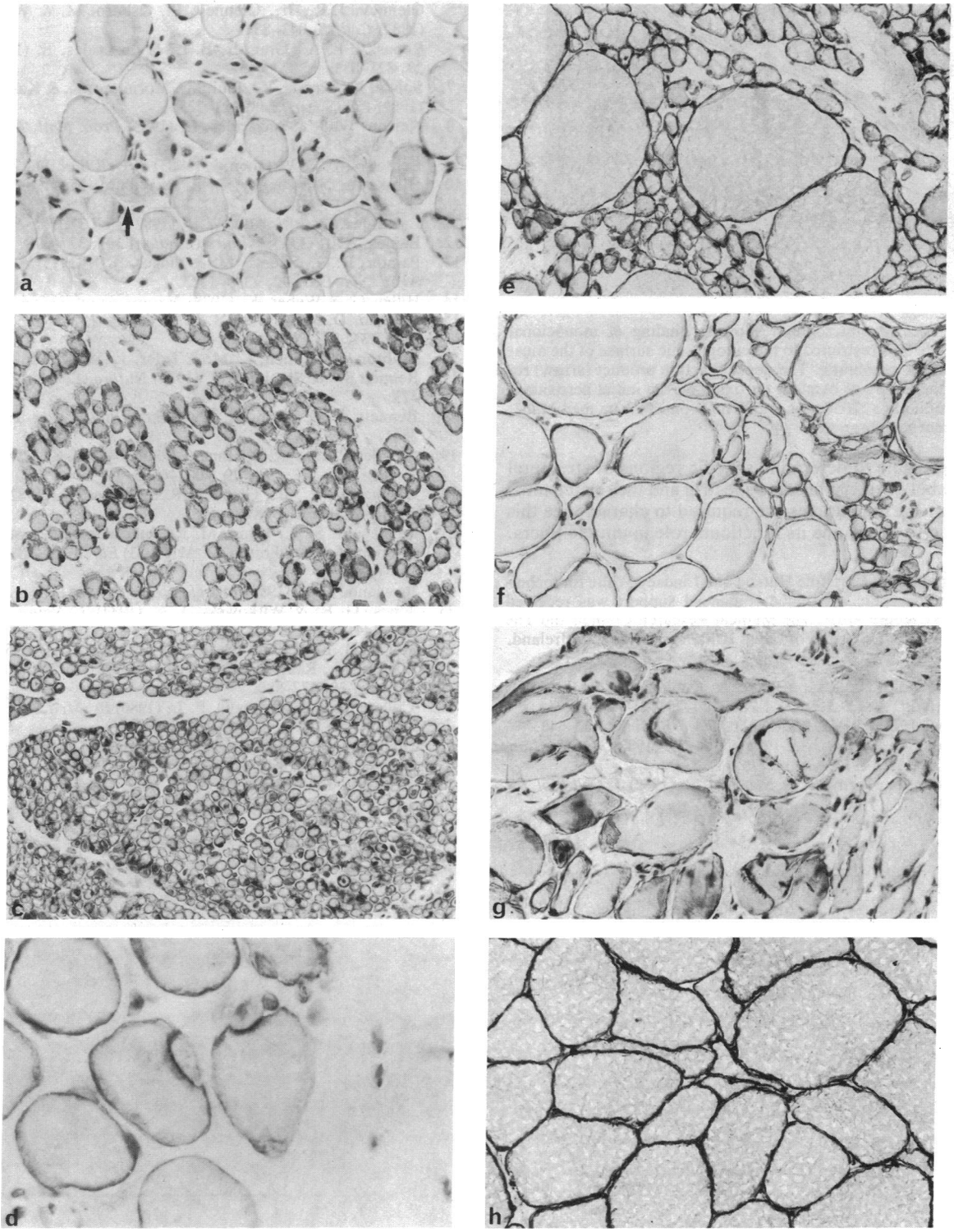

FIG. 2. Binding of monoclonal anti-spectrin antibodies to quadriceps muscle of patients with various neuromuscular disorders and to fetal muscle. (Anti-β-spectrin ascitic fluid 56A was diluted 1:1,000). (*a*) DMD; note weak antibody binding by regenerating fibers (arrow). (*b*) Fetal muscle, gestational age 17 wk; note strong binding of antibody. (*c*) Myotubular myopathy; note strong binding of antibody. (*d*) DMD; note lack of antibody binding by satellite cells but strong binding beneath them. (*e*) Spinal muscular atrophy. (*f*) Limb girdle muscular dystrophy; note split fiber. (*g*) Becker muscular dystrophy; note whorled fibers. (*h*) Peripheral neuropathy. Sections were counterstained with hematoxylin. (×220, except *d*, which is ×550.)

bers, but this was independent of any particular disease. It has been proposed (8) that in skeletal muscle, interactions between membrane and cytoskeleton, mediated by spectrin, may confer structural integrity on the myofibrils and plasma membrane during contraction. Increased antibody binding in diseased fibers might represent either a structural rearrangement of spectrin or an increase in its abundance in response to increased mechanical stress on the plasma membrane and myofibrils due to the effects of disease.

The high specificity of monoclonal antibodies to spectrin

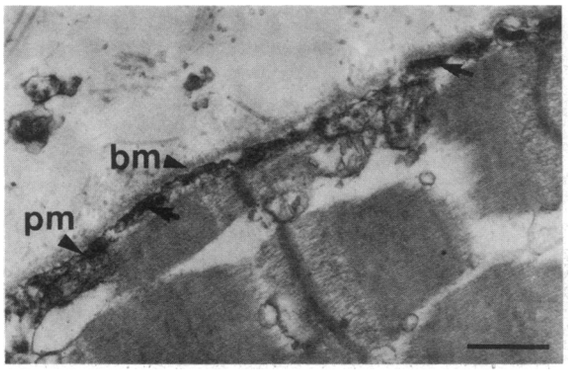

FIG. 3. Electron micrograph showing binding of monoclonal anti-β-spectrin 56A restricted to the cytoplasmic surface of the muscle fiber plasma membrane. The dense reaction product (arrow) results from the action of osmium tetroxide on the initial peroxidase reaction product. (×22050; bar = 500 nm.) pm, Plasma membrane; bm, basement membrane.

makes it possible to determine specific regions of structural homology between erythrocyte spectrin and the related muscle protein. Further studies are required to characterize this protein and to determine its functional role in muscle fibers.

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