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RAPID COMMUNICATION

Normal and Dystrophin-Deficient Muscle Fibers in Carriers of the Gene for Duchenne Muscular Dystrophy

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Dystrophin is the gene product that is affected in Duchenne muscular dystrophy (DMD). Antibodies against dystrophin were used to study the protein in muscle fibers of carriers of the gene. The results showed that DMD carriers have normal and dystrophin-deficient fibers. Dystrophin immunohistochemistry may be helpful for the detection of DMD carriers. (Am J Pathol 1988, 133:440–445)

DUCHENNE MUSCULAR DYSTROPHY (DMD) is the most common of the childhood dystrophies. It is inherited as an X-linked recessive disorder and the incidence is approximately 1 in 3000 live male births.[1] Recently, dystrophin, a newly recognized muscle-specific protein was identified as the affected gene product in DMD.[2] In electrophoretic studies, the protein was missing in patients with DMD and showed abnormal molecular weight in patients with Becker muscular dystrophy.[2,3] In immunohistochemical studies, dystrophin has been localized to the sarcolemma of normal muscle fibers. In DMD patients and in mice with X-linked muscular dystrophy (mdx), the sarcolemmal immunostain was either absent or markedly reduced in frozen sections of muscle samples.[4,5]

We have now studied dystrophin in DMD carriers using anti-dystrophin antibodies in combination with immunofluorescence. The results showed that DMD carriers have two populations of fibers: normal and dystrophin-deficient.

Materials and Methods

We have studied muscle biopsies from nine obligate carriers and two possible carriers of the DMD gene. Carriers were considered to be genetically obligate cases if they had two or more affected sons or one affected son and another affected maternal relative. Possible carriers were mothers of one isolated case of DMD. Four obligate and one possible carrier showed clinical evidence of proximal muscle weakness (manifesting carriers). Ages of the 11 women ranged from 19 to 63 years. Serum CK values ranged from 48 to 511 IU (normal < 50 IU). Muscle biopsies were frozen in liquid nitrogen-cooled isopentane, sectioned for diagnostic purposes, and stored in liquid nitrogen until

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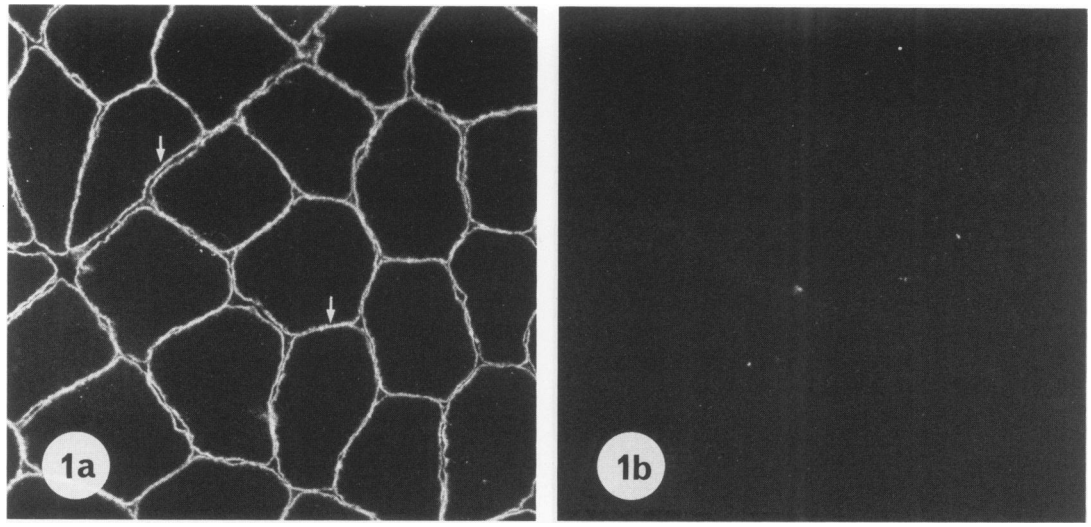

Figure 1—Binding of anti-dystrophin in normal human muscle. **a**—Immunostaining is seen at the sarcolemma (arrows) of all muscle fibers. **b**—Control section incubated with nonimmune serum shows lack of stain at the surface of the fibers. ×200

the time of this study. As controls, we used specimens from four women (ages 16, 20, 25, and 32 years) who, after full diagnostic studies at Columbia-Presbyterian Medical Center, were deemed to be free of neuromuscular disease. All human samples were obtained in accordance with the guidelines of the Columbia University Human Subjects Committee.

We used the original polyclonal antibodies of Hoffman et al[3] raised against fusion proteins (a gift of Drs. L. M. Kunkel and E. P. Hoffman). The sheep antisera against the 60 kd antigen used in this study reacted only with dystrophin in Western analysis of SDS-solubilized muscle tissue, indicating the monospecificity of the antibody preparation.[3,5]

For immunohistochemistry, 4 μ-thick sections from normal and DMD carrier muscle were placed on the same coverslip and incubated with anti-dystrophin diluted 1:1000 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). After 2 hours, the sections were washed 3 times in PBS, incubated for 20 minutes with biotinylated anti-sheep IgG (Amersham Corporation, Arlington Heights, IL; 10 μg/ml), and washed 3 times in PBS. The sections were incubated for 1 hour with streptavidin-fluorescein (Amersham Corporation; 10 μg/ml) (1:250), washed 3 times with PBS, and mounted with 50% glycerol in PBS.[5] Control sections were incubated with nonimmune serum and with PBS containing 1% BSA. To evaluate morphologic alterations of the muscle fibers, additional sections were stained with hematoxylin and eosin (H & E), modified trichome, and myofibrillar ATPase pH 9.4.[5] The sections were examined and photographed with a Zeiss II photomicroscope equipped with epi-illumination.

For evaluation of dystrophin immunostain at the sarcolemma, portions of five different fascicles from each sample were photographed using a ×16 objective. The negatives were then projected onto paper to determine the number of fibers surrounded by a continuous layer of immunofluorescence at the sarcolemma and the number of fibers with absent or markedly diminished immunostain at the cell surface. A total of 200–220 fibers were counted for each sample and the results were expressed as a percentage.

Results

In cross-sections of the four normal biopsies, dystrophin was localized at the sarcolemma of the fibers. The cell surface of each muscle fiber showed a thin and continuous layer of immunofluorescence and there was no immunostain of intracellular components (Figure 1a). We saw no staining of capillaries or interstitial cells and there were no muscle fibers that lacked dystrophin. Examination of control sections incubated with nonimmune serum showed no reaction at the sarcolemma (Figure 1b).

In frozen sections from the five manifesting DMD carriers, dystrophin immunostain was lacking or markedly reduced in a population of fibers that formed scattered small groups throughout the samples (Figure 2a). Except for small discontinuities of immunostain at the surface of occasional large fibers, the reaction at the sarcolemma was present and appeared continuous in the other muscle fibers. Adjacent sections from these samples stained with H & E showed marked variation in fiber size, increased central nuclei, occasional regenerating fibers, scattered groups of small muscle fibers, and

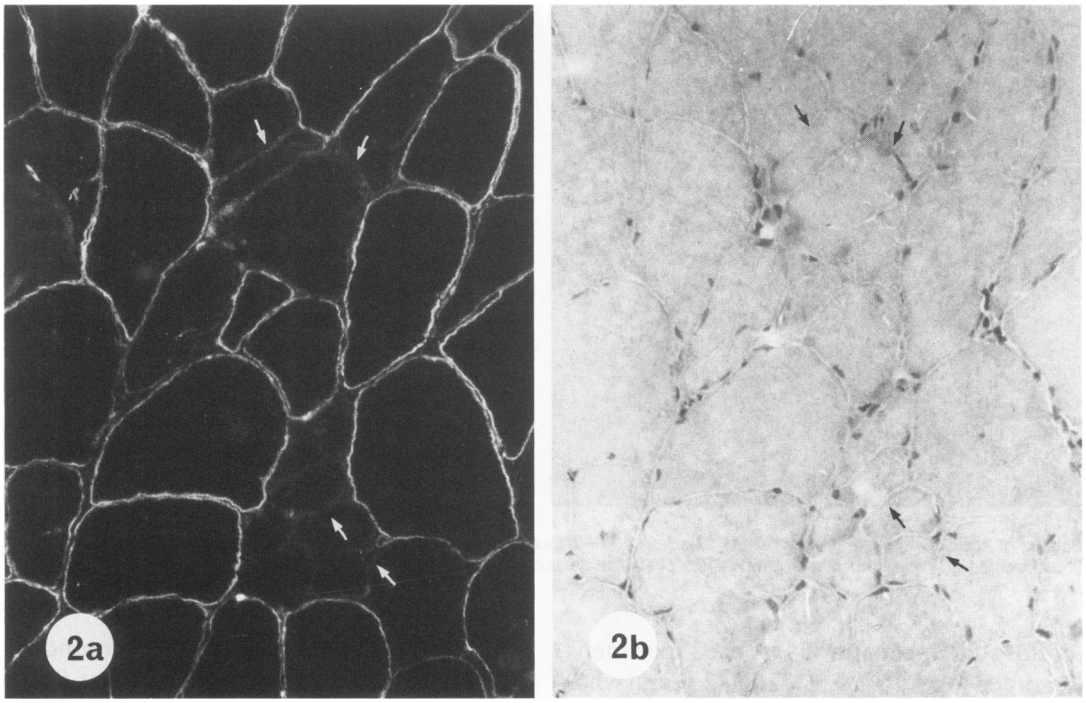

Figure 2—Binding of anti-dystrophin in a muscle section from a manifesting DMD carrier. **a**—The immunostaining at the sarcolemma is markedly reduced (arrows) in two groups of fibers. Although reduced the amount of immunofluorescence observed at the sarcolemma of these fibers appeared greater than the background staining noted in control sections incubated with nonimmune serum. **b**—Adjacent section stained with H-E shows that muscle fibers with reduced dystrophin (arrows) do not appear to be degenerating or regenerating. ×250

focal increase of connective tissue. The groups of small fibers did not appear to be regenerating or degenerating (Figure 2b) and with stains for myofibrillar ATPase pH 9.4, the dystrophin-deficient groups comprised both type I and type II fibers.

In frozen sections from four obligate but asymptomatic DMD carriers, there was lack or marked reduction of dystrophin immunostain at the sarcolemma of isolated muscle fibers (Figure 3a). Only rarely were these fibers adjacent, forming groups of two or three fibers (Figure 3b). Adjacent sections from these samples stained with H & E showed mild variation in fiber size, increase central nuclei, and occasional hyaline fibers.

In muscle sections from one obligate and one possible DMD carrier, no dystrophin-deficient fibers were

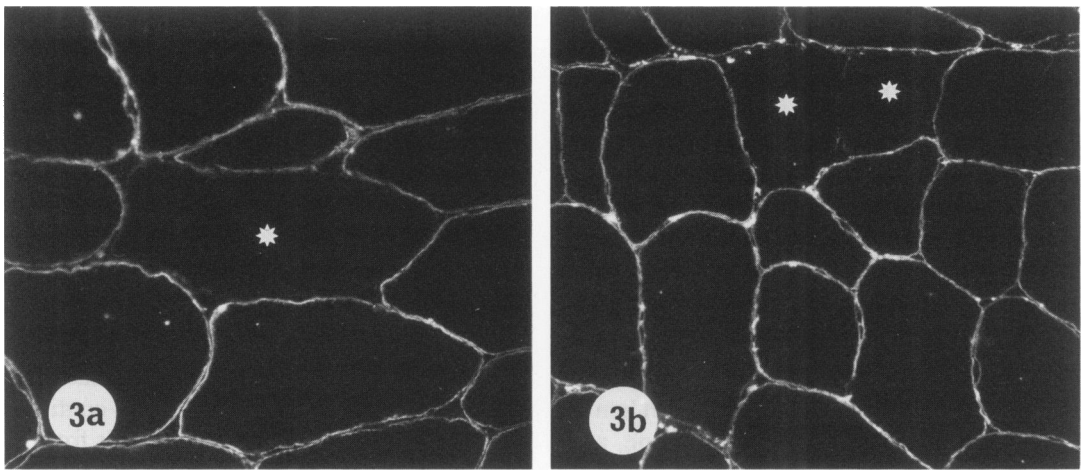

Figure 3—Binding of anti-dystrophin in a muscle section from an asymptomatic DMD carrier. **a**—One isolated fiber (star) shows lack of immunostain at the sarcolemma. ×260 **b**—Two adjacent fibers (stars) show reduced dystrophin at the sarcolemma. The faint immunofluorescence noted in these fibers was greater than the background seen in sections incubated with nonimmune serum. ×350

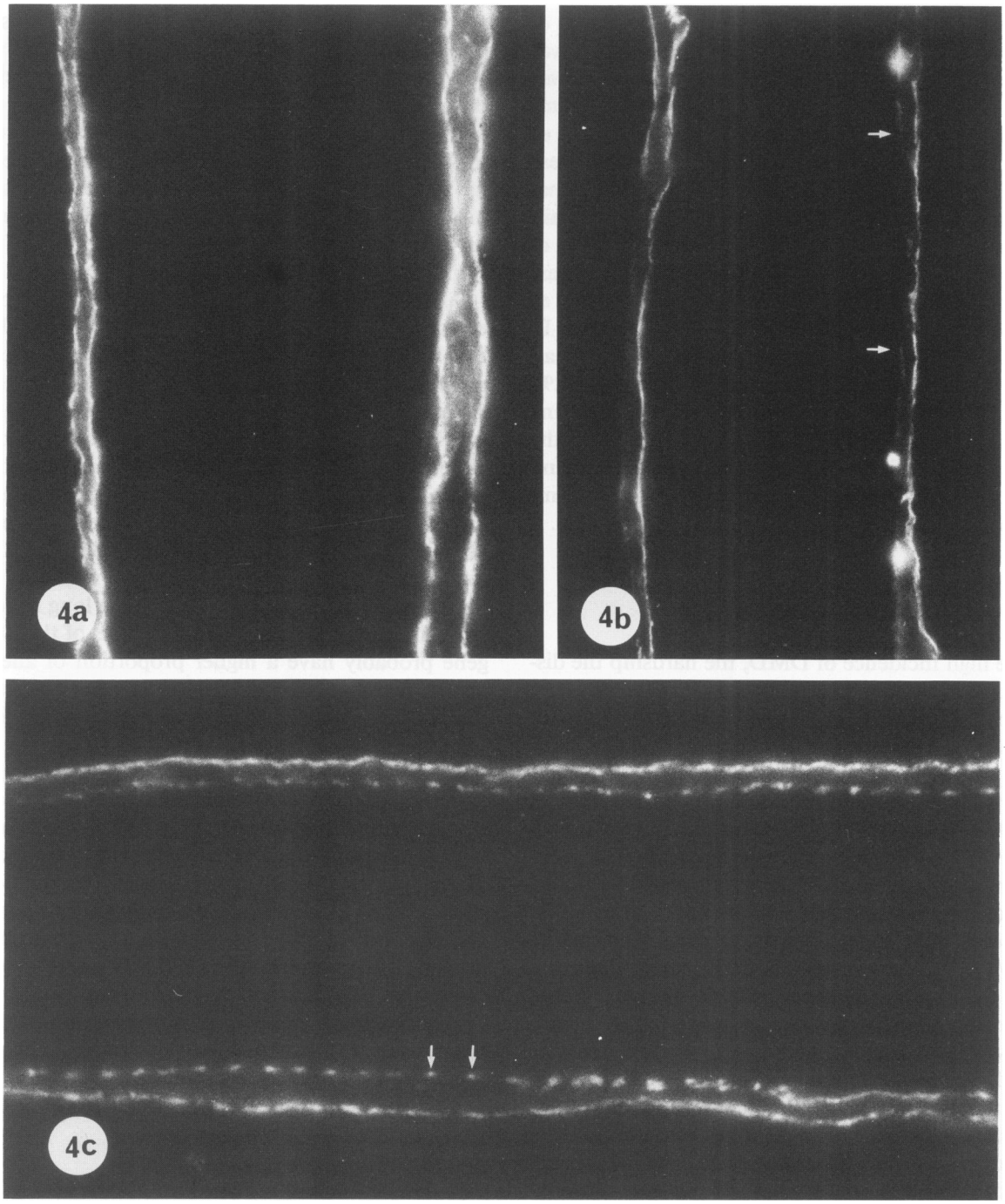

Figure 4—Binding of anti-dystrophin in longitudinal sections. **a**—Normal human muscle shows continuous immunostain at the sarcolemma. **b**—Section from a DMD carrier shows large gaps (arrows) in dystrophin immunostain. **c**—Another section shows (arrows) punctuated and discontinuous immunostain in one dystrophin-deficient fiber. ×2000

detected. Adjacent sections from these specimens stained with H & E showed mild variation in fiber size and few central nuclei.

Longitudinal sections from normal muscle showed continuous dystrophin immunostain at the sarcolemma of the fibers (Figure 4a). In manifesting and nonmanifesting DMD carriers the dystrophin-deficient fibers showed either large gaps in the immunostain (Figure 4b) or a punctuated and discontinuous immunostain of the sarcolemma (Figure 4c).

To evaluate the number of dystrophin-deficient fibers, we counted in cross-sections only those fibers showing lack, or marked reduction of immunostain (Figure 3a) and those in which more than 40% of the

sarcolemma remained unstained on map-reading of the projected negatives. The cutoff limit of 40% was chosen because rare fibers at the periphery of the samples in both carriers and normals showed gaps of immunostain that comprised up to 30% of the surface of the fiber. The invariably peripheral location of these fibers and the fact that they were seen in both normal and carriers suggest that these gaps of immunostain were due to mechanical damage during handling of the specimens. When the dystrophin-deficient fibers were counted and averaged for all 11 carriers, 12% of all fibers examined showed dystrophin deficiency. In individual carriers, the proportion of fibers with altered dystrophin immunostain ranged from 0–32%, and the proportion was higher in the five manifesting carriers (18.2, 22.4, 27.1, 28.3, and 32.2%) than in the six asymptomatic ones (0.0, 0.0, 2.1, 2.5, 4.1, and 4.3%). In the controls, fibers lacking dystrophin immunostain at the sarcolemma were never observed.

Discussion

The high incidence of DMD, the hardship the disease imposes, and the potential transmission by healthy females make detection of heterozygous carriers one of the major goals in the prevention of the disease.

For many years, tests used for identification of DMD carriers have included determinations of serum CK levels, electromyography, and morphologic evaluation of muscle biopsy.[6] These tests are far from perfect however, because they provide information about secondary phenomena, not the primary abnormality in DMD. Another difficulty in DMD carrier detection stems from the X-linked nature of the disease, because X chromosome inactivation results in greater variability of heterozygote phenotypes than in autosomal disorders. This variability of expression may facilitate carrier detection in those heterozygotes that show clinical abnormalities. On the other end, a proportion of carriers will be difficult to detect by traditional methods and will require tools capable of identifying a double population of muscle cells, or DNA analysis.[7,8]

This study used antibodies directed against the DMD gene product dystrophin to study the protein in individual fibers from carriers of the gene. In transverse sections of normal human muscle, the immunocytochemical reaction for dystrophin is evident in the sarcolemma of all muscle fibers.[4,5] In longitudinal sections, the protein is distributed homogeneously in the sarcolemma, all along the length of the fiber. In patients with typical DMD, the protein is either totally absent or there are only faint nonhomogeneous spots of immunoreactive material in the sarcolemma.[4,5]

We have now found that muscle from carriers of the gene contain two populations of fibers, some containing dystrophin and others with partial deficiency of dystrophin as evaluated by the criteria adopted by us. The proportion of dystrophin-deficient fibers was higher (18–32%) in manifesting carriers than in asymptomatic carriers, where the proportion of dystrophin-deficient fibers was 0–4.3%.

These results are compatible with the Lyon hypothesis stating that there is random inactivation of the X chromosome early in development.[9,10] In DMD carriers, the formation of myotubes is thought to result from the random fusion of two populations of myoblasts, one carrying the mutated X-chromosome, the other containing the normal X. The proportion of nuclei containing the mutated DMD gene within any muscle fiber of a carrier will determine whether the fiber will have a normal amount, partial deficiency or total lack of dystrophin at the sarcolemma. Our data provide graphic evidence of something long believed by clinicians—that manifesting carriers of the DMD gene probably have a higher proportion of affected muscle fibers than asymptomatic carriers. The results suggest that symptoms may result even when fewer than half of the fibers are affected.

Dystrophin immunohistochemistry may be useful for diagnosis, but we have not yet established specificity or reliability. It would be helpful if a simple histologic test of this kind were available, because although DNA diagnosis of the carrier state is currently the most reliable, this method is expensive, time-consuming, and not effective in all families.[7] Quantitative analysis of dystrophin must still rely on gel electrophoresis and this method may not be sensitive enough to detect a small population of dystrophin-deficient fibers. Further testing of the immunocytochemical method therefore seems warranted.

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