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From The New England Journal of Medicine, Pickard, N.A., Gruemer, H.D., Verrill, H.L., et al., Systemic Membrane Defect in the Proximal Muscular Dystrophies, Vol. 299, Page 841, Copyright © 1978 Massachusetts Medical Society. Reprinted with permission.

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The New England Journal of Medicine

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Volume 299

OCTOBER 19, 1978

Number 16

SYSTEMIC MEMBRANE DEFECT IN THE PROXIMAL MUSCULAR DYSTROPHIES

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Abstract We studied lymphocyte capping in 61 patients with Duchenne, Becker, limb-girdle, facioscapulothoracic and congenital muscular dystrophies. All showed a markedly diminished percentage of capped cells when compared with 86 normal controls, providing support for previous evidence that an alteration in membrane fluidity may be a common pathogenic feature in several genetically distinct forms of proximal muscular dystrophy.

Heterozygous carriers of Duchenne muscular dystrophy showed diminished capping that was indis-

tinguishable from that of afflicted males and was often present even when serum enzyme levels were normal. Studies in 25 families with 16 suspected sporadic cases indicated that no more than four out of 30 afflicted males may represent new mutations.

These findings imply that most cases of Duchenne dystrophy might be prevented by a population screening program for carrier females combined with prenatal detection of afflicted males. (N Engl J Med 299:841-846, 1978)

INCREASED serum activities of enzymes normally found in muscle have been the biochemical hallmark for the diagnosis of muscular dystrophy and for the detection of carriers of X-linked Duchenne muscular dystrophy. Some investigators have postulated that muscle-membrane permeability changes are responsible for the abnormal release of cytoplasmic enzymes from the cell interior into serum.¹⁻³ Recent evidence by transmission and scanning electron microscopy has demonstrated the existence of membrane surface alterations in the erythrocytes of patients with Duchenne-type dystrophy and their carriers as well as in other forms of proximal dystrophies.^{3,4} Additional proof of altered red-cell membrane conformation stems from findings of abnormal properties of membrane-bound enzymes in patients with Duchenne muscular dystrophy.^{5,6}

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Supported by a training grant (GM 01805) from the National Institutes of Health, by the Muscular Dystrophy Association, by an intramural grant from the Department of Pathology, Medical College of Virginia, and by the Roy E. Brownell Pediatric Research Fund of the Mott Children's Health Center, Flint, MI (excerpts have been taken from a dissertation submitted by Dr. Pickard to the Ohio State University Graduate School in partial fulfillment of the requirements for the degree of doctor of philosophy).

Conformational membrane changes may express themselves in altered membrane fluidity. The fluid mosaic model of membranes has been the subject of several reviews.^{7,8} Membrane fluidity may be assessed by electron spin resonance or more commonly by observation of the aggregation of fluorescent antigen-antibody complexes on the surface of B lymphocytes (capping phenomenon). Preliminary accounts of the applicability of these two techniques to the investigation of muscular dystrophy are already available.^{9,10}

The mechanism of cap formation, however, is far from understood, though certain cellular and subcellular components in lymphocytes and fibroblasts have been implicated in the process.¹¹⁻¹⁴ In brief, the capping technique permits one to observe the lateral mobility of integral membrane proteins in the plane of the cell surface. Normally, these membrane proteins are uniformly dispersed across the cell surface and are anchored by microtubules. In the presence of certain drugs as colchicine, or at low temperature, the microtubules depolymerize, and, when the temperature is raised above 4°C, the proteins are seen to move from the dispersed state through various stages of aggregation (clusters) to an asymmetric distribution of larger aggregates (patches) and finally into a large single aggregate that appears under the microscope as a fluorescent cap sitting on a cell surface. The same redistribution can be stimulated by the presence of polyvalent ligands (such as fluorescein-labeled immunoglobulins) when the cells are warmed to 37°C. The

roles of Ca^{++} , the membrane-associated contractile elements, the microfilaments and the anchoring microtubules in permitting specific cell membrane proteins to migrate to one pole of the cell are under active investigation. It may be assumed that a wide number of factors can bring about surface Ig-receptor-antibody mobility changes, including abnormalities in membrane conformation and composition, and in the availability of energy in the form of ATP.¹⁶⁻¹⁸ Thus, measurement of membrane protein mobility appears to be a sensitive indicator of a multitude of possible intracellular and associated cell surface changes.

The general availability of fluorescent microscopes and the relative ease with which studies of B and T lymphocytes may be carried out prompted us to apply the capping technic to the clinical and differential diagnostic investigation of the various forms of muscular dystrophy. When cells other than muscle fibers are examined, the underlying assumption is that any membrane abnormality found is of a systemic nature. Refinements in the recognition of conformational membrane changes should be useful in overcoming frequently encountered difficulties in carrier detection for genetic counseling.

MATERIALS AND METHODS

Isolation of Human Lymphocytes¹⁹

Fifteen milliliters of heparinized blood were collected, and the plasma separated by centrifugation for 10 minutes at $450 \times g$ for later biochemical analysis. The cells were resuspended in 23 ml of Seligmann's balanced salt solution (Grand Island Biological Company, Grand Island, New York 14072) and transferred into a 50-ml polycarbonate tube. Ten milliliters of a Ficoll-Paque solution (Pharmacia Fine Chemicals, Piscataway, New Jersey 08854) was layered beneath the cell suspension by a syringe fitted with a small-bore tube. Centrifugation of the samples ($450 \times g$ for 25 minutes at 25°C) resulted in four layers: erythrocytes, Ficoll-Paque, lymphocytes and Seligmann's balanced salt solution.

The lymphocyte layer was removed and placed into a 50-ml polycarbonate tube. The cells were washed with Seligmann's balanced salt solution, centrifuged and resuspended in the balanced salt solution, and any remaining erythrocytes were lysed with 9 ml of distilled water and within 10 seconds thereafter 2.0 ml of 0.6 mol per liter of sodium chloride was added. The cells were washed twice in the balanced salt solution ($250 \times g$ for 10 minutes) and finally resuspended in 5 ml of the solution and counted in a Coulter Model F counter. The final cell suspensions were greater than 95 per cent lymphocytes, with some other mononuclear and neutrophilic cells present. The yield of lymphocytes was about 90 per cent of the original suspension. This suspension of lymphocytes constituted the starting material for the B-cell and T-cell labeling procedure.

Labeling of B-Lymphocyte Surface Immunoglobulins

Approximately 4×10^6 lymphocytes were mixed with 0.1 ml of a 10-fold dilution of fluorescein-labeled polyvalent anti-human immunoglobulin (Meloy, Springfield, Virginia 22151) and incubated in the dark at 4°C for 30 to 60 minutes. The conjugated antiserum was diluted with 0.15 mol per liter of sodium chloride immediately before use. The optimum dilution was determined by a comparison of serial dilutions of the antiserum against the percentage of positively labeled cells; the largest dilution yielding maximum labeling was used. Low-temperature labeling was used to in-

hibit mobility of the membrane proteins. The cells were centrifuged ($200 \times g$ for 10 minutes at 4°C), washed three times in ice-cold Seligmann's balanced salt solution and resuspended in a few drops of cold Seligmann's balanced salt solution. The labeled cells were then removed from the low-temperature environment and placed in a 37°C water bath for 30 minutes. A wet-mount slide was prepared, and the lymphocytes were observed under visible and fluorescent illumination at magnification of 1250 times, a standard Zeiss microscope fitted with a VI FI epifluorescent condenser being used. At least 50 labeled cells were classified in the following categories: uniform, clustered, patched or capped distribution. Only the cells that had reached the capping stage were reported as a percentage of the total number of labeled cells because their distinct pattern virtually excluded a misidentification. In most cases, the microscopist was not aware of the identity of patient or control samples at the time of counting.

Labeling of T-Lymphocyte Concanavalin A Receptors¹¹

Approximately 4×10^6 lymphocytes were incubated with fluorescein-labeled concanavalin A (F-Con A, 100 μg per milliliter, Miles Laboratory, Incorporated, Research Division, Elkhart, Indiana 46514) in the dark at 4°C on a rotator for 15 minutes. The cells were centrifuged ($700 \times g$ for three minutes at 4°C), washed twice in cold Seligmann's balanced salt solution and resuspended in 1 ml of this solution. The cells were then incubated at 37°C for 30 minutes, a wet-mount slide prepared, and a minimum of 100 labeled cells classified as either uniform or capped. Only single cells and those in very small clumps (two to four cells) were counted for percentage of caps formed.

Patient Selection

Only patients who, in our judgment, had unequivocal clinical diagnoses were selected for this study. The evaluation was mainly based on personal and family history, neurologic examination, serum enzyme determinations and electromyography with nerve conduction studies. Fresh-frozen sections of muscle biopsies were stained with hematoxylin and eosin, Gomori trichrome, and histochemically for NADH (tetrazolium) dehydrogenase and ATPase at pH 4.5 and 10. In cases with a positive family history, some of the unaffected members also underwent a neurologic examination. Some of them had an electromyography and a muscle biopsy performed as well. In these cases the disease process was classified as one of the following neuromuscular disorders: Duchenne muscular dystrophy; Becker muscular dystrophy; facioscapulohumeral muscular dystrophy; limb-girdle muscular dystrophy; congenital muscular dystrophy; myotonic atrophy; Kugelberg-Welander disease; amyotrophic lateral sclerosis; and polymyositis.

Our study selectively included families in which the probands could be considered spontaneous mutations from available family history.

RESULTS

All 61 patients afflicted with Duchenne muscular dystrophy, Becker muscular dystrophy, limb-girdle muscular dystrophy, facioscapulohumeral muscular dystrophy or congenital muscular dystrophy demonstrated a quantitative reduction in the number of fluorescent cells that reached the capping stage, while showing no change in the percentage of cells that were labeled, indicating a normal B-lymphocyte count. The reduction in the frequency of capped cells is shown in Figure 1 and is compared with 86 normal controls, for whom a range of 46 to 68 per cent capping was obtained. In 30 patients from 25 families

with Duchenne muscular dystrophy, the range of cap formation was between 0 and 22 per cent, and seven members of one kindred with Becker-type X-linked dystrophy had values ranging from 20 to 40 per cent. Seven patients with facioscapulohumeral dystrophy had cap values between 4 and 14 per cent; the pedigree of a family with eight affected members is shown in Figure 2. B-lymphocyte capping from 16 patients with limb-girdle muscular dystrophy in nine families revealed a rather wide spread from 2 to 44 per

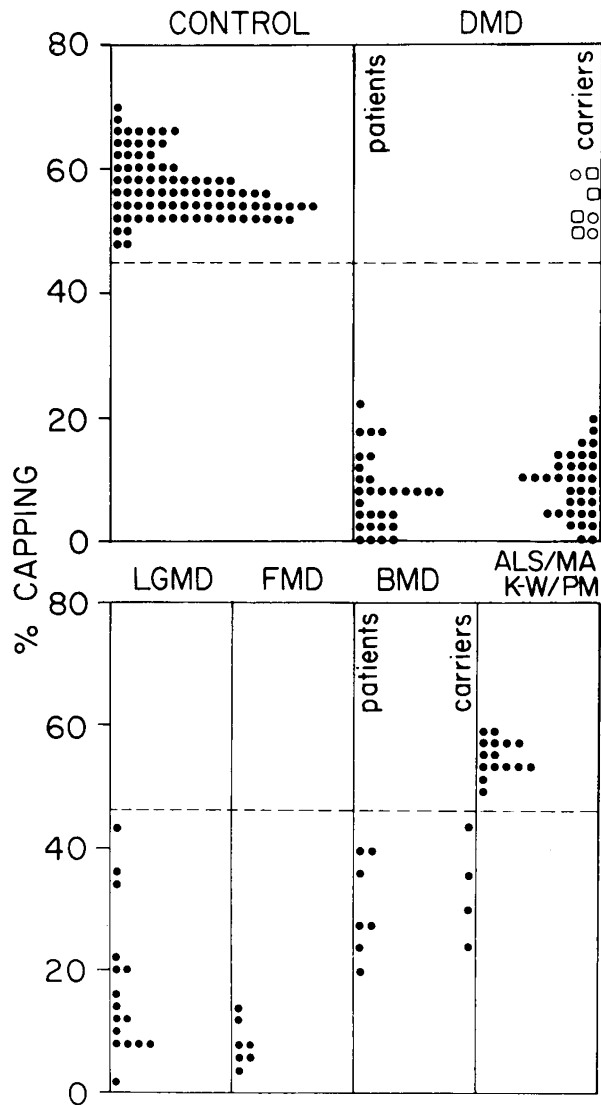


Figure 1. Distribution of Capping Results in B Lymphocytes. □ denotes carriers with normal capping, ○ carriers with normal capping and evidence of unequal X-chromosome inactivation in somatic cells, DMD Duchenne muscular dystrophy, BMD Becker muscular dystrophy, LGMD limb-girdle muscular dystrophy, MA myotonia, ALS amyotrophic lateral sclerosis, K-W Kugelberg-Welander disease, and PM polymyositis. The dotted line indicates the lower limit of the range of values observed among 86 normal controls.

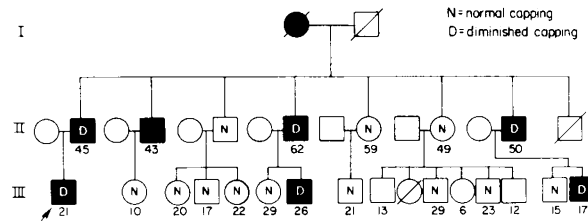


Figure 2. Pedigree of a Family with Facioscapulohumeral Muscular Dystrophy.

Affected members are indicated by black squares (males) or circles (females). Slashes through symbols denote deceased individuals. Arabic numbers represent ages, and the arrow designates the proband.

cent; unfortunately, the relatives of those affected were not available for establishing whether the mode of inheritance was autosomal recessive or dominant — information that might explain the heterogeneity of the expression at the membrane level. Normal capping was observed in six patients with myotonic atrophy, two with amyotrophic lateral sclerosis, four with polymyositis and two with Kugelberg-Welander disease (Fig. 1).

Of the 25 probands with Duchenne muscular dystrophy for whom detailed family histories were known, nine mothers were assumed to be carriers because of increased serum enzyme activities, and all nine exhibited decreased lymphocyte capping. In the remaining 16 cases, there was no family history of muscular dystrophy, and the probands, all male, could have represented new mutations or chance isolated familial cases. Although in all these 16 cases the maternal serum enzyme activities were within normal limits, the maternal B-lymphocyte cap formation was abnormally low in nine of them, falling within the range typical for obligate carriers of and patients with Duchenne muscular dystrophy (Table 1, families 1 through 9). In the last seven mothers lymphocyte capping was normal (families 10 through 16), but three of them in families 14, 15 and 16 each had a relative who exhibited low capping.

The family in Figure 2 demonstrates the usefulness and reliability of the test for identifying patients afflicted with autosomal dominant facioscapulohumeral muscular dystrophy. As may be seen from the pedigree, nonaffected members in the second generation had only healthy offspring and showed normal B-lymphocyte capping, whereas those afflicted with the disorder, if mated with normal persons, had the expected 50 per cent chance of producing afflicted children with diminished cap formation. Two clinically afflicted sons in the second generation and one in the third generation had normal serum enzyme activities, but all three had low capping values.

To demonstrate that the decreased capping of B lymphocytes in muscular dystrophy does not arise from any specific characteristic of the surface immunoglobulins of B lymphocytes, a technic was applied

Table 1. Enzyme and Capping Pattern in Mothers and in Sons Assumed to Have Been "Spontaneous Mutations."

FAMILY No.	SERUM ENZYMES IN MOTHER*	% OF LABELED CELLS CAPPED		
		MOTHER	AFFECTED SON	OTHERS
	AST, CK, LD			
1	Normal	4	4	
2	Normal	4	2	
3	Normal	6	0	
4	Normal	10	NT†	
5	Normal	2	NT	
6	Normal	6	0	
7	Normal	12	NT	
8	Normal	16	NT	
9	Normal	6	NT	
10	Normal	60	18	
11	Normal	54	12	
12	Normal	58	8	
13	Normal	60	NT	
14	LD HB	60	10	14 (mother) 8 (grandmother)
15	LD HB	54	8	10 (daughter) 54 (daughter) 56 (husband)
16	Normal	52	18	12 (daughter) 54 (husband)

*AST denotes aspartate aminotransferase, CK creatine kinase, LD lactate dehydrogenase, & HB high borderline.

†Not tested.

that labels membrane glycoproteins and involves predominantly T cells instead of B lymphocytes. With concanavalin A as the ligand, cap formation of lymphocytes from 12 patients and 10 carriers of Duchenne muscular dystrophy decreased to a range of 2 to 14 per cent, as compared with the range of 14 to 32 per cent in 14 healthy subjects.

No consistent age or sex dependence of lymphocyte capping was seen in the control, diseased or carrier populations; there was no difference in the percentage of B and T lymphocytes in patients or carriers of muscular dystrophy as compared to the control population, and the percentage of capped cells in both control and patient samples was usually well within a 10 per cent range upon repetition.

DISCUSSION

The results of this study are consistent with previous reports of a systemic membrane defect in red blood cells and in muscle tissue of patients with Duchenne, limb-girdle and facioscapulohumeral muscular dystrophy,⁴ and suggest that the defect includes B and T lymphocytes. These myopathies, as well as congenital muscular dystrophy and Becker-type dystrophy, both studied in this investigation, belong to a group of genetic disorders that, from a pathogenic point of view, appear to involve an inherited, systemic membrane defect. Furthermore, conformational membrane alterations are evidently responsible for the diminution of lymphocyte capping, even though the primary defect may be disease specific and several steps removed from the membrane itself. Presumably,

the systemic membrane defect is incompatible with normal muscle function and results clinically in one of the forms of muscular dystrophy.

From a clinical standpoint, the ability of the capping test to reveal membrane abnormalities in cells other than those undergoing degenerative changes makes this method useful in distinguishing the muscular dystrophies from conditions with similar clinical symptoms. Of particular concern in differential diagnosis is polymyositis. This disease is often manifested by muscle weakness similar in distribution to that seen in limb-girdle dystrophy and by abnormally high serum creatine kinase activity.

Patients with polymyositis and Kugelberg-Welander disease have normal lymphocyte capping and are thereby easily differentiated from those with the dystrophies (Fig. 1). Moreover, the normal capping test in myotonia atrophica suggests that this disease has a different pathogenesis from that of the proximal muscular dystrophies since the latter appear to be characterized by primary membrane abnormalities. The normal capping observed in lymphocytes from patients with myotonia atrophica does not exclude a membrane abnormality in this disease; in fact, erythrocyte membranes in this disorder have been shown to be abnormal.^{8,9} The capping test is at present of limited value in differentiating among patients with various forms of muscular dystrophy. It remains to be observed, however, whether differential diagnosis may be greatly aided if additional family members are available to permit determination of the mode of inheritance as, for example, between the autosomal recessive and the autosomal dominant form of limb-girdle muscular dystrophy. If the intermediate capping values observed in our one kindred with Becker muscular dystrophy can be further substantiated in other families, the capping technic may prove to be helpful in the differential diagnosis of the X-linked muscular dystrophies as well.

The data here are not meant to imply that the capping test reveals the severity of the cellular defect or of the dystrophy itself. Facioscapulohumeral and limb-girdle dystrophy progress somewhat more slowly than Duchenne-type disease, and yet all three diseases are manifested by lymphocyte capping values between 0 and 20 per cent; furthermore, nonafflicted carriers of Duchenne muscular dystrophy also exhibit the same range of values as affected males. Also, there is no apparent correlation between the severity of the disease and capping within a particular form of dystrophy. The only other disease known to have decreased lymphocyte capping is chronic lymphocytic leukemia.¹¹

Many X-linked traits show variable expression in female heterozygotes, a phenomenon that has frequently been attributed to the effects of random X inactivation.¹⁹ However, the data in Figure 1 give no hint that cap formation is more variable in the carriers of Duchenne muscular dystrophy than in the afflicted

males. To the contrary, decreased capping appears to be an all-or-none phenomenon in carrier females. This situation is not without precedents in X-linked traits. For example, except in unusual circumstances,²⁰ it has been difficult if not impossible to demonstrate cellular mosaicism in females who are heterozygotes for XgA.²¹

In 1935, Haldane²² estimated the rate of spontaneous mutation of the gene for hemophilia on the assumption that the number of genes lost by the failure of affected males to reproduce must equal the number of new mutations if the pool of defective genes is to remain stable. Since one third of the X chromosomes in the population occur in males, and Duchenne dystrophy is a genetic lethal, one third of the genes for this disorder are lost with each generation. Under the assumption that the mutation rates in males and females were equal, one third of the males afflicted with the disorder have previously been thought to be the result of new mutations. This view has generally been supported by the statistical estimates of the proportion of "sporadic cases" among affected males.²³⁻²⁶ Conflicting evidence has recently been provided by Roses et al., who used methods such as lactate dehydrogenase isoenzyme-5 determinations,²⁷ endogenous phosphorylation of peak II in red-cell membranes,⁶ and measurement of muscle protein synthesis²⁸ to detect carriers. The observation that there appear to be more carriers of the Duchenne trait among mothers of affected probands than the theory permits²⁷ cannot be reconciled with the assumptions of Haldane's analysis, and illustrates the potential importance of accurate carrier-detection tests for measuring mutation rates for X-linked genes.²⁹ The detection of spontaneous mutations can only be as accurate as the means for detecting carriers, and as newer, more sensitive carrier-detection methods are developed, estimates of new mutations in the hemizygotic sons must be adjusted. Our experience tends to confirm the observation of a low mutation rate by Roses and his co-workers. The mothers in Table 1, families 1 through 9, demonstrated a severe reduction in cap formation, indicating that they were indeed carriers for the Duchenne trait and, therefore, that their sons' affliction was not due to a spontaneous mutation in the mother. On the other hand, the last seven mothers in families 10 through 16 had normal lymphocyte capping and normal serum enzyme activities — findings that could have been interpreted as indicating that their sons represented new mutations except for the observations in families 14, 15 and 16. The mother of the proband in family 14, for instance, consistently demonstrated normal serum enzyme activities and normal cap formation, whereas her mother, grandmother, aunts and cousin had lymphocyte capping values below 15 per cent (Fig. 3). Since the probability of two or more spontaneous mutations occurring in one family is remote, unequal somatic X-inactivation seems to be the more likely explanation

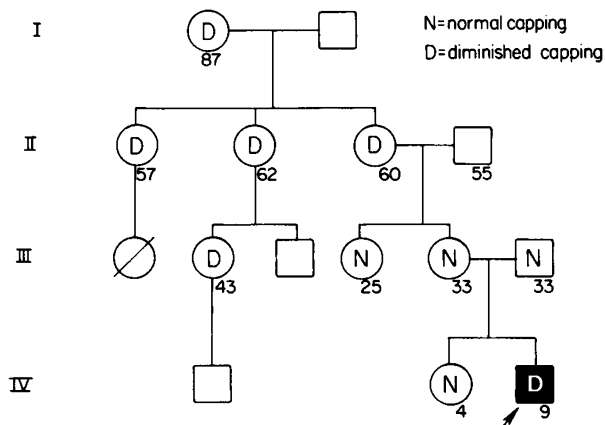


Figure 3. Occurrence of Unequal Somatic X-Inactivation (Lyon Hypothesis) in the Mother of the Proband of Family 14 in Table 1.

The proband is afflicted with Duchenne-type dystrophy. Symbols are explained in Figure 2.

for the normal findings in these three mothers. Previous evidence for the Lyon hypothesis in Duchenne dystrophy has been described in a pair of phenotypically discordant female identical twins.³⁰

Unequal somatic X-inactivation might also account for the absence of carrier findings in the other three mothers of the afflicted children of families 10 through 13. However, unless other carriers or afflicted males can be located in these kindreds, new mutation cannot be ruled out. Thus, if lymphocyte capping is used to detect carrier females, at most only four of the 30 cases appear to represent new mutations. The apparent discrepancy between the predicted and observed proportion of new mutations can be reconciled if the mutation rate in males is assumed to be higher than that in females. In his 1935 paper, Haldane²² derived a formula for the proportion, m , of affected males with an X-linked trait who carry new mutations. He gave $m = (1 - f)u / (2u + v)$, where u and v are the mutation rates in females and males and f the fitness, which is assumed to be 0 for a genetic lethal such as Duchenne muscular dystrophy. Setting $m = 4/30 = 0.133$ suggests that the mutation rate in males is 5.5 times that in females. This estimate is conservatively low since there was a conscious ascertainment bias for families with a single affected male. Among these families even more than one third would have been expected to represent new mutations if one third of all cases (both familial and sporadic) were assumed to arise by this mechanism. The apparent inequality in mutation rates may mean that the vast majority of affected males are born to carrier females, half of whom carry a new mutation, arising in the previous generation.³¹ The hypothesis of unequal mutation rates could be further tested by observations of the age and distribution of carriers in the maternal grandparents

of affected probands. If confirmed, these findings would imply that most cases of Duchenne dystrophy could potentially be prevented by a population screening program for carrier females combined with prenatal detection of affected males. However, as with any new test, the full range of conditions in which a false-positive result may be observed remains to be established.

Note added in proof: Additional studies in family 11 (Table 1) demonstrated low lymphocyte capping in the proband's maternal grandmother and aunt. The proband's mother therefore has to be assumed to show unequal X-inactivation, changing the proportion *m* in our population from 4/30 to 3/30, thus further increasing the calculated mutation rate of males over that of females to 8, if Haldane's equation is applied.

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