Absence of Correlation Between Skewed X Inactivation in Blood and Serum Creatine-Kinase Levels in Duchenne/Becker Female Carriers

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The pattern of X inactivation in lymphocyte DNA was investigated in 107 Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) carriers (102 asymptomatic and 5 manifesting carriers) and 117 normal female controls of different ages, with the aim: a) to analyze the pattern of X inactivation in blood DNA of a large number of DMD/BMD carriers as compared to normal female controls; b) to determine if there is a decrease in serum creatine kinase (CK) levels with age in obligate DMD/BMD carriers; c) to determine if there is a correlation between X-chromosome inactivation and serum CK among asymptomatic DMD/BMD carriers of different ages or with different clinical manifestations in symptomatic carriers. A high proportion of females showed extremely skewed X inactivation (>90% of one X preferentially inactivated), which was almost the same among carriers and normal controls (19 and 24%, respectively). The mean serum CK was significantly greater among young (<20 years old) than adult (>20 years old) DMD/BMD carriers and it decreased significantly until age 20 with an apparent stabilization afterwards. No statistically significant correlation was found between the proportion of active XN in blood and serum CK activity in DMD/BMD carriers although it was higher among those less than 20 years old. Our observations suggest that highly skewed X-chromosome pattern in blood (with preferential inactivation of the XN chromosome) is not enough to predict that a young DMD carrier will develop muscular weakness. Am. J. Med. Genet. 80:356–361, 1998. © 1998 Wiley-Liss, Inc.

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INTRODUCTION

Most Duchenne muscular dystrophy (DMD) symptomatic or asymptomatic carriers, with grossly elevated serum creatine-kinase (CK) have a normal X-chromosome constitution [Emery, 1963; Zatz et al., 1973, 1987]. Skewed X-chromosome inactivation, with preferential inactivation of the X chromosome bearing the normal dystrophin allele (XN) in most cells was suggested as a possible mechanism to explain such cases [Zatz et al., 1973; Emery, 1993; Isaacs and Badenhorst, 1987; Azofeifa et al., 1995]. In addition, it has been observed that symptomatic carriers with normal X-chromosome constitution may show a mosaic pattern of dystrophin-positive and negative fibers which might reflect the proportion of myonuclei bearing the active XN versus XDMD allele in muscle [Bonilla et al., 1988; Vainzof et al., 1991, 1993].

Several DMD-manifesting carriers may occur in the same family [Zatz et al., 1973, 1987; Moser and Emery, 1974] and this has also been observed in other X-linked disorders [Emery, 1993]. It has been suggested that if clinical manifestations are a consequence of skewed X inactivation, this familial concordance would suggest that X inactivation, at least in these women, may not be entirely random but is perhaps under genetic control [Emery, 1993].

More recently, the pattern of X-chromosome inactivation in DMD carriers was analyzed at the molecular level by different groups of investigators [Bushby et al., 1993; Pegoraro et al., 1994, 1995; Tihy et al., 1994; Azofeifa et al., 1995; Matthews et al., 1995]. However, these investigators analyzed only a small number of females or focused mainly on “manifesting” carriers.

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However, little is known about the correlation between serum CK levels and the pattern of X-chromosome inactivation in a larger sample of young and adult DMD asymptomatic carriers.

In affected male DMD/Becker muscular dystrophy (BMD) patients, it was shown that the activity of CK in serum gradually decreases with advancing age due to the progressive elimination of dystrophic fibers [Pennington, 1980; Rowland, 1980; Edwards et al., 1983; Hoffman et al., 1987, 1988; Zatz et al., 1991]. It was suggested by us and others, that the serum CK activity in female heterozygotes seems to decrease also with age due to the same mechanism observed in affected boys [Dreyfus et al., 1966; Thompson et al., 1967; Lange and Zatz, 1979; Zatz and Otto, 1980; Zatz et al., 1980, 1998; Passos-Bueno et al., 1989]. However, in the premolecular era it was not possible to identify among young “at-risk” females those who were obligate carriers of the DMD mutated gene.

Therefore, the present investigation was undertaken in order : a) to analyze the pattern of X inactivation in blood DNA from a large number of DMD/BMD carriers as compared to normal female controls ; b) to determine if there is a decrease in serum CK levels with age in obligate DMD/BMD carriers whose carrier status was confirmed at the molecular level; c) to verify if there is a correlation between X-chromosome inactivation and serum CK among asymptomatic DMD/BMD carriers of different ages or clinical manifestations in a small group of symptomatic carriers.

SUBJECTS AND METHODS

All subjects were ascertained at the Centro de Miopatias, Universidade de São Paulo, Brazil. The classification of DMD/BMD carriers was based on family history, high serum CK levels and DNA analyses which included: screening of deletions in the dystrophin gene, linkage analysis [Passos-Bueno et al., 1990; 1992] and screening of point mutations by SSCP and sequencing as reported previously [Sitnik et al., 1997]. Serum CK activities were determined as previously reported [Zatz et al., 1976, 1998].

A total of 151 asymptomatic DMD/BMD carriers whose carrier status was confirmed at the molecular level had their serum CK levels determined (Table I). Serum CK was determined in at least two different samples but in most the final values corresponded to the mean of three or more independent blood collections (collected with intervals of at least one week).

<table>
<thead>
<tr>
<th>Age range (years old)</th>
<th>Number</th>
<th>Mean serum CK (Su/mL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–10</td>
<td>17</td>
<td>107.1 ± 126.1</td>
</tr>
<tr>
<td>11–20</td>
<td>21</td>
<td>40.5 ± 70.6</td>
</tr>
<tr>
<td>21–30</td>
<td>32</td>
<td>15.1 ± 14.32</td>
</tr>
<tr>
<td>31–40</td>
<td>47</td>
<td>16.7 ± 12.64</td>
</tr>
<tr>
<td>41–50</td>
<td>24</td>
<td>22.5 ± 24.23</td>
</tr>
<tr>
<td>51–70</td>
<td>10</td>
<td>8.7 ± 7.7</td>
</tr>
</tbody>
</table>

*Normal values: young girls (mean age 6.1 ± 3.7, n = 100), 7.0 ± 6.6 Su/mL; adult women (mean age 28.8 ± 7.3, n = 383), 5.1 ± 3.3 Su/mL.

Among them, 102 who were heterozygote for the androgen receptor (AR) locus (and in whom the two alleles could be clearly discriminated) were investigated for the pattern of X inactivation in DNA from peripheral blood. In addition, five manifesting DMD carriers were also included in the present investigation.

In 89 among the 102 asymptomatic carriers and in the five manifesting carriers, it was possible to identify the X carrying the normal allele (XN) and that bearing the DMD mutated allele (XDMD). The identification of the XN and XDMD was performed through the use of several polymorphic markers along the X chromosome (in order to exclude the possibility of recombination between the AR and the DMD/BMD loci).

The control group included 117 normal female relatives of patients with no X-linked diseases and normal volunteers. All studies were done after informed consent.

For X-inactivation analyses, DNA was extracted from peripheral blood, and 200 µg of digested and non-digested DNA samples (digested with Alu I and Cfo I ) were used as templates for amplification of the AR highly polymorphic (CAG)n repeat as reported in Edwards et al. [1992] and Allen et al. [1992].

All samples were run in duplicate in a 5% polyacrylamide gel (19:1 acrylamide:bis-acrylamide). A densitometer Shimadzu CS-9000 was used to determine the ratio of X inactivation in each sample and the mean of two readings was considered for each case. Since one allele may amplify more than the other a correction factor was applied to compensate for unequal amplification of alleles. As reported previously [Pegoraro et al., 1994], this was done for each patient, calculating first the ratio between the two alleles of the undigested DNA and correcting the final values for preferential polymerase chain reaction amplification. The degree of X inactivation in the digested DNA was calculated by normalizing the sum of allele A plus allele B to 100%.

In addition, in order to assess the statistical error for the percentage of inactivation of each allele, we took a random sample of 10 individuals and for each one of them the analysis was repeated in different replicas. The result of this analysis showed that the standard errors of the mean ranged from 0.005 to 0.025 with coefficients of variation ranging from 1.1 to 9.8%. The average values of these two parameters (respectively 0.0145 and 3.8%) were taken as representative of the standard error and the coefficient of variation for each measurement, that is, the average value of two estimated percentages from each individual.

The X-inactivation pattern (that is, the proportion of cells with either X inactivated) was classified as skewed X-chromosome inactivation, when more than 80% of one X chromosome was preferentially inactivated [Harris et al., 1992] and extreme skewed chromosome X inactivation when more than 90% of one X chromosome was preferentially inactivated.

RESULTS

X-Chromosome Inactivation Pattern and Frequency of Skewing

The pattern of X-chromosome inactivation in 117 normal control females showed that 42/117 (36%) had a
skewed pattern (>80%) and 28 (24%) had more than 90% of one X chromosome preferentially inactivated.

The pattern of X-chromosome inactivation in 102 asymptomatic DMD/BMD female carriers showed a distribution similar to the one observed in normal females: 35 of them (about 34%) had a skewed pattern (>80%) and 19 (about 19%) had more than 90% of one X chromosome preferentially inactivated.

Analysis of Age Effect in CK Level of DMD/BMD Female Carriers

As seen in Table I, on average the greatest values were observed in young girls from 1 to 10 years old and there was a decrease in mean serum CK levels until age 20 with an apparent stabilization afterwards. Statistical analysis showed that the mean serum CK was significantly greater (t = 3.35; P < 0.01) in the group of carriers younger than 20 (CK = 72.96 ± 102.85 Sigma units (Su); n = 38) than in the group older than 20 (CK = 16.75 ± 16.23 Su; n = 113).

X-Chromosome Inactivation Pattern and Serum CK Levels in Asymptomatic DMD/BMD Carriers

In 89 DMD/BMD asymptomatic female carriers for which it was possible to determine the inactivation pattern of the X chromosome bearing the XN or XDMD allele, 33 showed skewed X-chromosome inactivation (>80%), with a similar proportion of either X inactivated: 15 had a preferential inactivation of XN and 18 showed a preferential inactivation of the XDMD (P >0.05). Furthermore, among female carriers who had extreme inactivation of the XDMD or, on the other extreme, of the XN alleles, there was a similar proportion of females with increased serum CK: four among seven with >90% of the XN inactivated versus four among eight with >90% of the XDMD inactivated.

No statistically significant correlation was observed between the mean serum CK and the percentage of XN preferentially inactivated X chromosome (R = 0.14; P >0.05) when all females were analyzed together. In order to assess if such correlation might differ in younger as compared with older DMD carriers we repeated this analysis dividing the females into two groups: younger than 20 (Group a) and older than 20 (Group b) since serum CK activity was shown to be significantly higher in Group a than in Group b. As seen in Figure 1, there was a stronger correlation between the mean serum CK and the percentage of XN preferentially inactivated among younger (<20 years old, r = 0.42) than older females (>20 years old; r = 0.08) but it still did not reach the level of significance (P = 0.054 and P = 0.489, respectively).

Manifesting Carriers

Among the five patients DMD manifesting (Table II), two belonged to families with DMD-affected patients (Cases 1 and 3) and three were isolated cases (2, 4, and 5). All had increased serum CK activities (on average 14-fold above normal). Among the isolated cases, Patient 2 had a t(X; 22) translocation (with the breakpoint at Xp21), Patient 4 had a deletion (Δ4) in the dystrophin gene, but no deletion was detected in Patient 5.

Three among the five patients had extreme (100%) X-chromosome skewing including the patient with the t(X; 22) translocation. Dystrophin analysis showed a negative pattern in the two severely affected girls (including the X/22 translocation).

DISCUSSION

X-Inactivation and Skewing in Normal Females

In the somatic cells of female mammals, dosage compensation for X-linked genes is achieved by random inactivation of either the maternally or paternally derived X chromosome (X<sup>M</sup> or X<sup>p</sup>) which occurs early in development and subsequently is stably inherited. Although the underlying mechanism is still unknown, the X-inactivation center (XIC) has been identified as a critical part of the X-inactivation process. It has been implicated in recognizing the number of X chromosomes and initiating a signal which propagates in cis, silencing most of the genes on the associated X chromosome [Plenge et al., 1997]. The XIST gene, which maps to the XIC region, is expressed exclusively from the inactive X chromosome and has been considered an
excellent candidate for the initiation or establishment of the inactivation signal [Brockdorff et al., 1991; Brown et al., 1991]. As a result of this process, most females have mosaic expression of maternal and paternal alleles of X-chromosome loci and the mean contribution from each X chromosome in somatic tissues is ~50%.

However, a skewed pattern of X-chromosome inactivation in blood leukocytes from normal females, ranging from about 10 to 33%, has been reported by several investigators [Nance, 1964; Gale et al., 1991, 1992; Fey et al., 1992; Brown and Brown, 1993; Harris et al., 1992; Puck et al., 1992; Pegoraro et al., 1994; Belmont et al., 1991; Busque et al., 1996; Naumova et al., 1996]. In the present sample of normal females, about 36% had a skewed pattern (>80%) and 24% showed extreme skewing (>90% one X preferentially inactivated). Although this frequency is apparently high, it is unlikely that our results are due to technical artifact since duplicate analysis showed good reproducibility. These results are consistent with the findings of Gale et al. [1991] and Fey et al. [1992] who also found a high frequency of skewing (23% and 33%, respectively, in normal blood leukocytes) suggesting that a higher proportion of the normal population may have a more skewed Lyonization pattern than previously reported. Thus, the present data show, in another population and using a different methodology, that a skewed X-chromosome pattern in blood is not rare, a finding for which we still have no explanation. Plenge et al. [1997] have recently identified a promoter mutation in the XIST gene in two unrelated families with skewed X-chromosome inactivation. However, this mutation is apparently rare and therefore could not explain the high proportion of normal females with a skewed X-inactivation pattern.

**Serum CK in DMD Carriers**

The observation that serum CK decreases with age in young carriers [Thompson et al., 1967; Moser and Vogt, 1974; Lange and Zatz, 1979; Passos-Bueno et al., 1989; the present study] has suggested that this might occur due to the progressive elimination of dystrophic fibers which contribute to the release of serum enzymes to the blood. However, before DNA analysis it was not possible to determine if a female was an obligate carrier of the DMD gene before the birth of an affected child. The present study confirms that although there is a wide variability in CK activities among individuals, the mean serum CK is significantly increased in DMD/BMD carriers younger than 20 than among those who are older than 20 (P < 0.01).

This observation supports the hypothesis according to which abnormal dystrophin-negative fibers segments in DMD carriers are gradually replaced by normal segments in local cycles of necrosis and regeneration [Matthews et al., 1995; Pegoraro et al., 1995]. If CK is released from dystrophin-negative muscle fiber segments, the replacement by normal segments will result in a decrease of serum CK levels.

**X-Inactivation Pattern in Asymptomatic and Manifesting DMD/BMD Carriers**

No significant correlation was observed between serum CK levels and the frequency of XN preferentially inactivated when all females were analyzed together. However, since serum CK was shown to be significantly higher in young than in adult DMD carriers one might expect to find a significant correlation between serum CK and skewed X inactivation only among females younger than 20 years old. Indeed, there was a stronger positive correlation between the mean serum CK and the percentage of XN preferentially inactivated in females younger than 20 than among those older than 20 but it was still not statistically significant, which might be explained due to the sample size. Therefore, it would be of interest to try to replicate this analysis in other population studies since previous reported studies on X-inactivation pattern focused mainly on manifesting DMD/BMD carriers but little is known about asymptomatic carriers.

On the other hand, reports on symptomatic DMD/BMD carriers showed some inconsistent results. Pegoraro et al. [1994] studied 13 female dystrophinopathy patients (ten isolated and three with a positive history of DMD in males) and observed that all had a skewed X-inactivation pattern in peripheral blood DNA, with preferential inactivation of the XN. In a subsequent study of 19 DMD carriers, these authors [Pegoraro et al., 1995] found that 9 of 14 skewed inactivation patients (12 isolated and 2 with a positive history of DMD) showed moderate to severe symptoms while the other five who had a mild phenotype were still young. In contrast, all five random inactivation patients showed a mild or asymptomatic phenotype. They suggest that the clinical picture for the skewed X-inactivation patients was more severe than for the random inactivation cases and that more than 90% of patients with skewed X inactivation in blood will develop

### Table II. Data From Manifesting Carriers

<table>
<thead>
<tr>
<th>Case</th>
<th>Familial data</th>
<th>Mutation</th>
<th>Karyotype</th>
<th>CK increased</th>
<th>Age</th>
<th>Muscular weakness</th>
<th>% of X inactivated in blood</th>
<th>% of Dystrophin-negative fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Familial (DMD nephew)</td>
<td>NI*</td>
<td>NI</td>
<td>4-fold</td>
<td>42</td>
<td>Moderate</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Isolated</td>
<td>NI</td>
<td>t(X:22)</td>
<td>9-fold</td>
<td>13</td>
<td>Severe</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Familial (2 DMD sons)</td>
<td>Δ51</td>
<td>NI</td>
<td>9-fold</td>
<td>33</td>
<td>Moderate</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>Isolated</td>
<td>Δ44</td>
<td>NI</td>
<td>23-fold</td>
<td>23</td>
<td>Moderate</td>
<td>67</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>Isolated</td>
<td>—</td>
<td>46.XX</td>
<td>28-fold</td>
<td>7</td>
<td>Severe</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

*NI, Not investigated

b) Vainzof et al. [1991; 1993].
a moderate to severe muscular dystrophy. In another study, Azofeifa et al. [1995] observed that in 8 among 11 symptomatic DMD carriers the manifesting phenotype could be explained by a preferential X-chromosome inactivation. However, in the remaining three there was no clear correspondence among highly skewed X-inactivation ratios and clinical symptoms or with the percentage of dystrophin-positive fibers. In another study, Matthews et al. [1995] investigated muscle X-inactivation patterns and dystrophin expression in the muscle of five asymptomatic females heterozygous for deletions in the dystrophin gene and five manifesting carriers (where only one had no family history). They observed that the pattern of X inactivation in muscle DNA was nonbiased in the five asymptomatic carriers but among the five manifesting carriers both highly skewed (90:10) and nonbiased patterns of X inactivation were found.

As seen in Table II, in the present study, three of the five manifesting carriers (including the X/22 translocation Case 3) had extreme skewing with a total inactivation of the XN in peripheral blood (Cases 1, 2, and 5). Patients 2 and 5, who have a Duchenne-like presentation had also a negative pattern for dystrophin. However, Patient 1, who also had total skewing in blood, is only moderately affected at age 42 and had only 14% of dystrophin-negative fibers in muscle biopsies. In addition, no correlation between X-chromosome pattern in blood and clinical signs was observed for the remaining two moderately affected manifesting carriers (Cases 3 and 4), who showed nonskewed X inactivation. A normal X-inactivation pattern in lymphocytes and muscle was also reported in a manifesting carrier by Bushby et al. [1993].

In addition, a considerable difference in X-inactivation patterns in different tissues of the same subject has been reported [Brown et al., 1990]. Azofeifa et al. [1996] compared the X-chromosome activation ratios among tissues of different embryonal origin, that is, leukocytes, muscle, thyroid gland, and medulla of the suprarenal glands. They found high intranidividual divergencies among tissues of different embryonal origin. According to Matthews et al. [1995] the pattern of X inactivation can vary not only between different tissues but even between different skeletal muscles from the same subject with no consistent relationship between the patterns of X inactivation and the proportion of dystrophin-negative fibers in carriers.

In summary, taking into account the relatively high frequency of skewed X inactivation observed in blood from normal females as well as the discordant X-inactivation pattern in different tissues from the same individual, the lack of a clear correlation observed by us and others in the X-inactivation ratios in blood and serum CK levels, clinical signs, or the proportion of dystrophin-negative fibers is not surprising. Thus, the finding of a highly skewed X-chromosome pattern (with preferential inactivation of the XN chromosome in blood) in a young sister of a DMD patient is not indicative that she will develop muscular weakness.

Furthermore, we have observed that not only DMD-manifesting carriers (or male patients affected with BMD) but also patients affected with severe autosomal recessive limb-girdle muscular dystrophy (sarcoglycaneopathies) may have a reduction in dystrophin as a secondary effect [Vainzof et al., 1996]. Therefore, the finding of reduced dystrophin in addition to a preferential inactivation of the XN allele in blood in a suspected female isolated case is not enough to confirm a diagnosis of dystrophynopathy and should be interpreted with caution.

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