The Effects of Dystrophin Gene Mutations on the ERG in Mice and Humans

Gerhard W. Cibis,*†§ Kathleen M. Fitzgerald,*† David J. Harris,‡
Paul G. Rothberg,‡ and Mahendra Rupani§

Purpose. The authors' earlier findings of a negative electroretinogram (ERG) in a boy with Duchenne muscular dystrophy (DMD) led them to investigate dystrophin gene deletions and ERGs in five boys with DMD. The authors wanted to determine whether there were similar ERG findings in an animal model for DMD, the mdx mouse.

Methods. Ganzfeld ERGs were recorded in five boys with DMD after a complete ophthalmic examination. The dystrophin gene was analyzed by Southern blot hybridization. ERGs were recorded in anesthetized mdx and control mice with a modified Grass photostimulator (Grass Instrument Company, Quincy, MA).

Results. Ophthalmic examinations in all five boys had normal findings, yet an abnormal negative ERG was recorded for each subject. The subjects' gene deletions were variable, ranging from large deletions to no detectable deletions. The ERGs of the mdx mice were normal and did not differ significantly from those of the control mice.

Conclusions. The authors believe the unique ERG recorded for the human subjects is a manifestation of DMD associated with defects at the dystrophin gene locus and represents a new clinical entity. The ERG of the mdx mouse may be spared for several reasons, including milder effects of the mouse gene defect, differences in muscle and retinal gene product, or species differences in the biochemical role of dystrophin. The ERG shows promise of becoming a noninvasive diagnostic tool for DMD and its milder allelic forms. Invest Ophthalmol Vis Sci. 1993;34:3646-3652.

Duchenne muscular dystrophy (DMD) is an X-linked recessive neuromuscular disease that leads to death in the second to third decades of life.1 Mutations that alter the structure or expression of dystrophin can cause either severe DMD or milder allelic forms, such as Becker muscular dystrophy. Dystrophin is a large (427 kD) protein with an amino acid sequence similar to the spectrin family of membrane cytoskeletal proteins. In humans, dystrophin is found throughout the body in smooth muscle but is most abundant in skeletal and cardiac muscle, where it has been localized to the inner face of the plasma membrane. Dystrophin is thought to stabilize the plasma membrane during muscle fiber contraction. The brain expresses significant levels of nonmuscle dystrophin, and approximately 30% of patients with DMD also have mental retardation.

Recently, negative electroretinograms (ERGs) have been reported in boys with DMD. An ERG is negative when the b-wave amplitude is less than the a-wave amplitude. The first patient, described by Weleber, Pillers, Powell et al, was a 6-year-old boy who had features of both Åland Island eye disease and incomplete congenital stationary night blindness (CSNB). This child had a deletion of part of band 21 of the short arm of the X chromosome (Xp21) and three other X-linked disorders: glycerol kinase deficiency, congenital adrenal hypoplasia, and DMD. The ERG showed negative scotopic and abnormal photopic waveforms, leading the authors to suspect that Åland Island eye disease and incomplete CSNB were the same entity. Later linkage studies by Alitalo, Kruse, Forsius, Eriksson, and Chapelle showed that the
Aland Island eye disease gene in the original Aland Island family was not in the region deleted in the patient described above. In this patient, the proximal end of the deletion was within the DMD locus.

Mutation of the dystrophin gene also accounts for muscle disease in the mdx mouse, an animal model for human X-linked DMD. Unlike humans, the mouse exhibits a mild nonprogressive muscle disease because of an early muscle destruction with subsequent regeneration. The gene mutation in the mdx mouse has been reported as a single base substitution within an exon; the translation of the dystrophin polypeptide is terminated at 27% of its length.

To determine the localization and functional significance of dystrophin, Miike, Miyatake, Zhao, Yoshio, and Uchino and Miyatake, Miike, Zhao et al suggested that dystrophin plays an important role in the physiological or structural role of the conduction system; however, it was not believed to be a critical role because there were no reports of apparent retinal abnormalities at that time. Bulman, Pillers, Weleber et al, aware of the recent findings of negative ERGs in DMD, investigated the normal human retina for the presence of dystrophin and found two isoforms in the outer plexiform layer of the retina. Dystrophin was absent from these regions in mdx mice. This raises the question of the functional role of dystrophin in the retina. Miyatake, Miike, Zhao et al suggested that dystrophin plays a role in the generation of the b-wave of the human ERG.

The role of nonmuscle dystrophin in the human retina is not known. To our knowledge, there have been no investigations into the presence of dystrophin in human DMD retina. Because dystrophin was shown to be completely absent in the mdx mouse retina, we decided to study ERGs in this species. In this article, we report the data from the mdx mouse and five boys with DMD and discuss the relationship between the two.

MATERIALS AND METHODS

Human Subjects

The subjects were recruited from The Children's Mercy Hospital Departments of Ophthalmology and Genetics in accordance with the tenets of the Declaration of Helsinki and after Internal Review Board approval. The ages of the five male subjects, ranged from 2 years, 4 months to 5 years, 10 months (mean, 4 years, 5 months). These subjects met the standard criteria for DMD: muscle weakness, elevated serum creatine kinase, a positive family history when the history was known, or consistent muscle biopsy specimens. A complete eye examination was performed before the ERG. A blood sample also was drawn for DNA analysis. The ERGs were compared with those of age- and gender-matched normal subjects. Previously recorded ERGs of subjects with CSNB were pulled from our database for comparison with those of our subjects with DMD.

Human ERGs

All the children underwent a complete ophthalmic examination. ERGs were recorded in accordance with published guidelines within the limitations of our recording system. The pupils of the eyes tested were dilated with 1.0% tropicamide and 2.5% phenylephrine hydrochloride drops. The patients' eyes were dark adapted for 45 minutes. Children who could not comply with the ERG protocol were sedated with 50 to 75 mg/kg oral chloral hydrate syrup. The cornea was anesthetized with 0.5% proparacaine hydrochloride, and the contact lens electrode was inserted under dim long-wavelength illumination. The eyes were tested simultaneously.

After dark adaptation, stimulus flashes of short-wavelength (blue) (Wratten filters 47, 47A, and 47B in combination, Eastman Kodak Company, Rochester, NY), long-wavelength (red) (Kodak Wratten 26), and white (xenon) were presented in a Ganzfeld bowl (Nicolet Biomedical Instrument Corporation, Madison, WI) under scotopic conditions. Flash luminance was attenuated with internal strobe settings and neutral density filters (Wratten). The recordings were obtained under photopic conditions with a rod-desensitizing background field of 10 fl to isolate the cone response. The stimulus flash was white (xenon). Flash and background luminance were calibrated with a photometer (model 350, United Detector Technology, Hawthorne, CA).

The ERGs were recorded with a monopolar "jet" contact lens electrode (Universo SA, La Chaux-De-Fonds, Switzerland) referenced to the patient's ipsilateral mastoid with a forehead ground. Recordings were performed with a Nicolet CA-1000 signal-averaging system (Nicolet). The conventional ERGs were recorded with a bandpass frequency setting of 1 to 1500 Hz (−3 dB points). Oscillatory potentials were recorded with bandpass frequency settings of 30 to 1500 Hz (−3 dB points). Responses were stored on floppy disks for later analysis.

The ERGs were recorded for a series of stimuli, first for the dark-adapted eye at 15-second intervals. One blue flash (−1.00 log cd-sec/m²), one red flash (0.66 log cd-sec/m²), and two separate white flashes (first, the conventional ERG [1.72 log cd-sec/m²]; second, the oscillatory potential [1.72 log cd-sec/m²]) were recorded at the filter settings described earlier.
The eyes then were light-adapted to a rod-desensitizing background of 20 fL for 10 minutes. The light-adapted stimuli were presented on a 10 fL background and consisted of one white flash (1.72 log cd-sec/m²) followed by a 30-Hz flickering white light (0.79 log cd-sec/m²).

Human DNA Analysis

DNA was isolated from the patient’s peripheral blood and analyzed by Southern blot hybridization with standard published methods. The genomic DNAs were digested with the restriction enzyme HindIII according to the manufacturer’s instructions (New England Biolabs, Beverly, MA), electrophoresed on a 1% agarose gel, and probed with fragments of the dystrophin cDNA. The dystrophin cDNA clones cDMD1–2a, cDMD2b–3, cDMD4–5a, cDMD5b–7, cDMD8, and cDMD9–10 were obtained from the American Type Culture Collection. Radioactive probes were generated by random primer labeling of DNA fragments purified by gel electrophoresis as described previously. Deletions in the dystrophin gene were evaluated by comparison of the autoradiographic patterns with normal controls and the published maps for this gene.

Animal Subjects

Ten control mice (C57 Bl/10 snj, five male, five female) and ten mdx mice (C57 Bl/10 ScSn-mdx/J, five male, five female) (Jackson Labs, Bar Harbor, ME) underwent an ERG at 2 and 5 months of age. The mice were used and sacrificed according to the ARVO Resolution on the Use of Animals in Research.

Animal ERGs

Recordings were performed with a Nicolet CA-1000 signal-averaging system, and the results were stored on floppy disk for later analysis. Whenever possible, we duplicated the techniques described by Heckenlively, Winston, and Roderick for recording the ERG. A DTL microfiber electrode was the active electrode, referenced to a Grass E2 platinum subdermal needle electrode (Grass Instrument Company, Quincy, MA) behind the ipsilateral ear. A needle electrode also was used for the ground, placed subcutaneously above the right leg.

The eyes were dilated with 10% viscous phenylephrine, and the mice were placed in a dark room for 45 minutes. After dark adaptation, the mice were anesthetized with an intraperitoneal injection of 0.0075 mg/g xylazine (1 mg/ml) and 0.075 mg/g ketamine hydrochloride (10 mg/ml). A drop of normal saline was instilled to prevent corneal drying. The electrodes were placed on the animal, and the ERGs were recorded under dim red illumination.

The stimulus was provided with a Grass PS-22 photostimulator. The face plate of the strobe was removed and replaced with an aluminum funnel. A 1 mm diameter fiber optic cable was attached to the end of the funnel and positioned 4 cm from the eye, delivering a 45-cd-sec/m² flash at intensity 116. The stimulus was presented at a rate of 1 Hz. The recordings were obtained with a 1 Hz low-pass filter and a 250 Hz high-pass filter. The analysis time was 200 milliseconds. Ten responses were averaged and stored on floppy disk. Two trials of ten averages were obtained for reproducibility. The b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. The b/a amplitude ratio was obtained by dividing the b-wave amplitude by the a-wave amplitude, measured from the baseline to the trough of the a-wave.

RESULTS

Human Subjects

The clinical features are summarized in Table 1. Eye examinations in all subjects showed minor refractive errors in two children: AR: -1.00 OD, -1.25 +0.50 X90 OS; BS: +1.00 +0.75 X90 OD, +1.00 +0.25 X90 OS. There were no abnormalities of the cornea, anterior chamber, iris, lens, vitreous, optic disk, retinal vessels, macula, or peripheral retina. The subjects had no visual complaints. Their parents did not observe behavior associated with night blindness.

Human ERGs

Figure 1 shows the ERGs of the five subjects with DMD compared with a normal 4-year-old boy. There were several morphologic differences that made the DMD ERG unique.

Under scotopic conditions, the response to a dim blue flash was highly attenuated or absent. The scotopically balanced response to a deep red stimulus showed only preservation of the cone contribution.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>mdx</th>
<th>t test†</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-time</td>
<td>16.5</td>
<td>16.1</td>
<td>0.1264</td>
</tr>
<tr>
<td>b-time</td>
<td>34.5</td>
<td>34.8</td>
<td>0.5759</td>
</tr>
<tr>
<td>a-amp</td>
<td>138</td>
<td>140</td>
<td>0.8746</td>
</tr>
<tr>
<td>b-amp</td>
<td>310</td>
<td>305</td>
<td>0.8204</td>
</tr>
<tr>
<td>b/a ratio</td>
<td>2.3</td>
<td>2.2</td>
<td>0.4966</td>
</tr>
</tbody>
</table>

There is no significant difference between groups.

* Mean values.
† Two-tailed.
ERGs in DMD and the *mdx* Mouse

Normal mate; age 4 years  DMD  BS; age 4 years 8 months  DMD  BE; age 2 years 4 months  DMD  BR; age 4 years 10 months  DMD  PM; age 4 years 4 months  DMD  AR; age 5 years 10 months

**Scotopic conditions:**

- 30 Hz Flicker: 0.79 log cd-sec/m²

**FIGURE 1.** The ERG on the left side of the figure is that of a representative normal male, age 4 years. Following are the ERGs of the five subjects with DMD. The DMD ERG, compared with those of age- and gender-matched normal subjects, shows a severely attenuated or absent b-wave to dim blue, red, and bright white stimuli under scotopic conditions. Oscillatory potentials are attenuated. The response to white stimuli under photopic conditions is missing the second oscillatory potential before the b-wave (arrow). Scotopic conditions = 45 minutes of dark adaptation with no background light. Photopic conditions = 10 minutes of light adaptation with 10 Fl background light. Dotted line represents stimulus onset.

Implicit times of these components were within normal limits.

Of particular interest is the negative response to the bright white stimulus under scotopic conditions. Oscillatory potentials were present on the ascending b-wave. The b-wave eventually returned to the baseline at approximately 150 milliseconds. When oscillatory potentials were isolated with a 30- to 1500-Hz bandpass in our normal subjects, the O2 peak was the most dominant. In subjects with DMD, the O1 peak was greatest in amplitude; the O2 peak was highly attenuated, and the O3 peak was absent.

After 10 minutes of light-adaptation, the DMD response to a bright white stimulus under photopic conditions also showed a missing oscillatory potential. In normal subjects we routinely recorded an O1, O2, and b-wave, but the O2 oscillatory potential was missing in our DMD subjects. Responses to 30-Hz flicker were not significantly different from normal.

**Animal Subjects**

Representative ERGs from control and *mdx* mice at the 5-month test period are shown in Figure 2. The large oscillatory potentials were attributed to the use of a 250-Hz high-pass filter. Table 2 includes the mean ERG implicit time, amplitude, b/a ratio, and results of two-tailed t-tests for both groups.

The ERG in the *mdx* mouse, unlike those in human

**FIGURE 2.** The ERGs of representative control and *mdx* mice at the 5-month test period. This response can be compared with the human ERG to bright white stimuli under scotopic conditions. A negative ERG was not seen in *mdx* mice at either the 2- or 5-month test date. Ten responses were averaged, stored on floppy disk, repeated, and superimposed to show reproducibility.
TABLE 2. Clinical Features of DMD Subjects

<table>
<thead>
<tr>
<th>Age at Diagnosis</th>
<th>Weakness</th>
<th>Pseudo-hypertrophy</th>
<th>Serum Creatine Kinase (U/L)</th>
<th>EMG</th>
<th>Deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 yr 8 mo</td>
<td>+</td>
<td>+</td>
<td>16,650</td>
<td>Abnormal</td>
<td>No detectable</td>
</tr>
<tr>
<td>2 yr 4 mo</td>
<td>+</td>
<td>+</td>
<td>9,449</td>
<td>Normal</td>
<td>Exons 9–44</td>
</tr>
<tr>
<td>4 yr 10 mo</td>
<td>-</td>
<td>-</td>
<td>23,450</td>
<td>Not done</td>
<td>Exons 47–52</td>
</tr>
<tr>
<td>4 yr 4 mo</td>
<td>+</td>
<td>+</td>
<td>47,000</td>
<td>Abnormal</td>
<td>Exons 47–746</td>
</tr>
<tr>
<td>5 yr 10 mo</td>
<td>+</td>
<td>+</td>
<td>27,000</td>
<td>Abnormal</td>
<td>Exons 49–51</td>
</tr>
</tbody>
</table>

DMD subjects did not show a negative response. A two-tailed t-test showed no significant difference between the groups for implicit time, b-wave amplitude, or b/a ratio. To assure ourselves that the ERG did not deteriorate with age, we reevaluated the ERG at 5 months. There was no difference between or within groups for the 2- and 5-month tests.

DISCUSSION

In this article, we describe a negative ERG in boys with DMD who had normal findings on ophthalmologic examinations and no functional complaints. Our data suggest this unique ERG is a manifestation of DMD associated with defects at the dystrophin gene locus and not another nearby gene. We believe the negative ERG associated with DMD is a new clinical entity. Several other retinal disorders are characterized by a negative ERG, including retinal dystrophies, vascular disorders, and retinal toxic effects.

The ERG in DMD, although similar to the negative ERG seen in forms of CSNB, seems to have enough differences to distinguish between the two (Fig. 3). Most notable are the oscillatory potentials.
ERGs in DMD and the mdx Mouse

seen under photopic conditions. These oscillatory potentials are absent in CSNB. Only the second oscillatory potential is absent in our subjects with DMD. Weleber and Eisner\(^\text{27}\) reported O2 to be small or absent in some normal subjects; however, this would be a rare finding in our laboratory because of the higher intensity of our stimulus. The absence of the photopic oscillatory potentials in CSNB has been the subject of several investigations\(^\text{28-30}\) into the on- and off-responses of the photopic ERG. Alexander, Fishman, Peacheys, Marchese, and Tso\(^\text{31}\) believed the abnormal rod and cone ERG in CSNB could be accounted for by a defect in synaptic transmission between photoreceptors and depolarizing bipolar cells. We are investigating the on- and off-responses of the DMD photopic ERG.

From this study, we believe the ERG in DMD represents a defect in signal transmission from the rod photoreceptor to the rod bipolar cell. This is consistent with the absent b-wave and present a-wave of the dark-adapted ERG. Because our subjects are so young, we have not been able to conduct psychophysical tests of dark-adaptation or to test rhodopsin levels by fundus reflectometry; however, given the long history of DMD and intense scientific investigations into the disease, it would be surprising indeed if a clinical manifestation as significant as night blindness had been overlooked. We report the association of an ERG that appears to show diminished rod function in a patient population with a seemingly benign ophthalmic history. If dystrophin does play an important physiologic or structural role in the conduction system, as Miyatake, Miike, Zhao et al\(^\text{14}\) suggest, is it necessary for generation of the b-wave of the ERG? Our findings of a normal ERG in the mdx mouse suggest it is not.

Why does the mdx mouse have a normal ERG, whereas humans with DMD have an abnormal ERG? Several possibilities exist. The mdx mouse has a milder muscular disorder for reasons that are not yet clear, and it is possible that the manifestation of the dystrophin gene defect in retina is similarly mild. This would predict that some humans with the milder allelic muscular disorder, such as Becker muscular dystrophy, would have a less severely affected ERG. This prediction is yet untested. It is also possible that the retinal dystrophin gene product is different from the muscle product because of alternative RNA splicing and the point mutation in the mdx mouse does not alter the retinal gene product. Perhaps species differences in the exact biochemical role of dystrophin may allow less of an impact from a lack of dystrophin function on the mouse retina as it does on the human retina.

Additional investigations of DMD and other neuromuscular diseases with electrophysiologic and psychophysical tests are necessary. It is important to identify the role of dystrophin and other related proteins in the outer plexiform layer of the retina. The ERG abnormality may reflect the severity of the muscle disease and, therefore, would show promise of becoming a noninvasive diagnostic and prognostic tool for DMD/Becker muscular dystrophy.

**ADDENDUM**

After this manuscript was accepted for publication, the authors became aware of new mdx mouse mutations with a different expression of muscle and nonmuscle dystrophin from the original mdx mouse.\(^\text{31}\) ERGs were recorded in the new mutations mdx2Cv, mdx3Cv, mdx4Cv and were found to be normal when compared to control mice.

**Key Words**

Duchenne muscular dystrophy, dystrophin, electoretinogram, mdx mouse, negative electoretinogram

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