Electroretinographic Abnormalities in Parents of Patients With Leber Congenital Amaurosis Who Have Heterozygous GUCY2D Mutations

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Background: Leber congenital amaurosis (LCA) is an infrequently encountered congenital form of retinitis pigmentosa with marked genetic and clinical heterogeneity. Thus far, 10 genes have been identified in this disorder since 1996. In the future, LCA may become treatable by gene and/or pharmacological intervention, and these therapies will likely be gene specific, giving major significance to rapid gene identification and gene-phenotype studies.

Objective: To test the hypothesis that parents of patients with LCA have identifiable electroretinographic and psychophysical changes.

Subjects, Materials, and Methods: Complete eye examinations and electroretinographic studies were performed on 2 sets of parents whose offspring were diagnosed as having LCA and who were found to carry a mutation in 1 of the 10 LCA genes—GUCY2D. One set of parents also underwent static perimetry threshold measurements.

Results: We found that single flash-light–adapted a- and b-wave amplitudes, 30-Hz flicker, or both cone signals were significantly decreased in amplitude in 4 heterozygotes, while 2 parents showed delayed 30-Hz flicker implicit times. Electroretinographic rod-mediated signals were normal in 2 of the heterozygotes, but subnormal in 2. Static perimetry testing showed normal thresholds in the 2 heterozygotes tested.

Main Outcome Measures: Single flash-light–adapted a- and b-wave amplitudes and implicit times, 30- or 32-Hz flicker amplitudes and implicit times, rod-mediated signals, and dark-adapted, rod-mediated thresholds.

Conclusions: Some carrier parents of patients with LCA and a GUCY2D mutation develop measurable, cone possibly rod abnormalities most consistent with a mild cone-rod dysfunction. This correlates well with the known retinal expression pattern of GUCY2D, which is considerably higher in cone compared with rod photoreceptor cells.

Arch Ophthalmol. 2002;120:1325-1330

LEBER CONGENITAL amau- rosis (LCA) (MIM 204000) is a congenital, retinal blind- ing disease with a worldwide prevalence of 3 in 100000 neonates. It accounts for 5% or more of all inherited retinal degenerations and for approximately 20% of the children attending schools for the blind around the world. Leber congenital amaurosis was described by Theodor Leber over 130 years ago as a congenital form of retinitis pigmentosa (RP), and represents one of the most severe forms of inherited retinal diseases. It is defined and characterized by severely impaired vision in the first 6 months of life, sensory nystagmus, poorly reactive pupils, and severely diminished or non-detectable cone-rod photoreceptor responses on the electroretinogram (ERG) in the first year of life. Leber congenital amaurosis is genetically heterogeneous, and since 1996, 10 separate genes have been implicated in the cause of LCA. In the future, LCA may be treatable by pharmacological intervention and/or gene replacement therapy, and these therapies will likely be gene specific, giving major significance to gene identification and gene-phenotype studies.

The first causal LCA gene discovered was the gene for retinal guanylate cyclase, GUCY2D, which encodes a transmembrane enzyme found in rod-cone outer segment disc membranes. Light exposure initiates the rod-cone phototransduction cascades, which results in the activation of β-phosphodiesterase, and hydrolysis of cyclic guanosine monophosphate (cGMP). This results in the closure of cGMP-gated ion channels, and a decrease in intracellular calcium concentration that, in turn, activates the enzyme guanylate cyclase–activating protein (GCAP). Guanylate cyclase–activating protein activates retinal guanylate cyclase, which replenishes the...
level of cGMP to its prelight exposure levels, and restores the opening of the cGMP-gated ion channels. Mutant retinal guanylate cyclase probably results in abnormally low levels of cGMP and permanent closure of the channels, a situation that corresponds to chronic light exposure.8 GUCY2D mutations have been found in recessive LCA,6,20,21 as well as in dominant cone-rod degeneration.22

We identified mutations in the GUCY2D gene in patients with LCA,20 and studied their effects in a cell culture system.23,24 We observed that the mutations negatively influence the wild-type allele in vitro (dominant negative effects). These findings prompted us to test the hypothesis of in vivo dominant negative effects by using ERG and psychophysical analyses of parents of children with LCA who have a GUCY2D mutation. We report significant and repeatable cone ERG abnormalities in parents of patients with LCA.

SUBJECTS, MATERIALS, AND METHODS

GENOTYPING

Family 1

As part of an ongoing evaluation of LCA phenotypes and genotypes, we are studying 250 patients with LCA from around the world. Mutation analysis of all known LCA genes (GUCY2D, RPE65, CRX, AIPL-1, TULP-1, CRB-1, RPGRIP-1, and LRAT) is routinely performed in our laboratory by polymerase chain reaction, single-stranded conformational polymorphism.20 Single-stranded conformational polymorphism variants are further analyzed by direct nucleotide sequencing, and/or by automated sequencing on an ABI prism 377 (Applied Biosystems, Warrington, England). To confirm mutations, we perform DNA-based diagnostics by restriction enzyme digests and exclusion in 100 ethnically matched control patients. Informed consents were obtained for venous blood sampling, according to the guidelines from the McGill University Montreal Children's Hospital's ethical review board.

Family 2

The proband of this family was part of another large mutation screening of all known LCA genes at the University of Iowa, Iowa City, by polymerase chain reaction, single-stranded conformational polymorphism, and automated sequencing, as previously documented.21 The parents were genotyped by denaturing high-pressure liquid chromatography at the University of Alabama at Birmingham as part of an ongoing parallel study.

ELECTROPHYSIOLOGICAL ASSESSMENTS

Detailed eye examinations, including Snellen visual acuities, slit-lamp biomicroscopy, and dilated indirect ophthalmoscopy were done on all parents. Electroretinograms were done on both eyes and performed in accord with a standard recommended by the International Society for Clinical Electrophysiology of Vision23,24 using procedures previously described by Peachey et al.26 In this article, we analyzed the ERGs of both parents of the child with a leucine to proline substitution (L954P mutation) in GUCY2D (family 1). Previously, we identified and reported this mutation in the proband with LCA and her mother (subject 1), but were unable to identify a GUCY2D mutation in the proband’s father (subject 2).20 We determined, in vitro, that the mutation causes a complete loss of retinal guanylate cyclase activity and inability to respond to GCAP (C. L. Tucker, PhD, V. Ramamurthy, PhD, A. L. Pina, PhD, unpublished data, June 2000).23

We compared our ERG findings from our heterozygous carriers to the range of normative data, which include ERG average values and 2 SDs above and below the mean (our reference range). We also sought to determine whether these ERG changes could be observed in LCA carriers in whom we were unable to identify mutations in the GUCY2D gene. We, therefore, performed ERGs on 12 additional parent pairs of offspring with LCA, according to procedures previously reported by Hébert et al.27 In these patients with LCA, we screened, but were unable to identify, pathogenic mutations in GUCY2D, RPE65, CRX, or AIPL-1.

Electroretinograms were also performed on another set of parents of a patient with LCA who also harbors a single mutation in GUCY2D (family 2). A mutation predicting a proline to leucine substitution (P575L) was found in the proband who had LCA and his mother (subject 3), while no GUCY2D mutation was found in the proband’s father (subject 4) (Figure 1). These ERGs were done in Rome, Italy, according to a previously reported procedure.28 For these ERGs, monopolar Henkes’ type contact lens electrodes were used to record the responses. Up to 40 responses were averaged off line for each testing condition. Maximal ERG responses and cone responses were recorded with a flash at the low end of the standard range (about 1.5 candela [cd]-s/m2), which is the standard intensity for this laboratory. A set of transient cone-driven ERGs was also recorded at approximately 3.5 cd-s/m2 (same as the University of Illinois at Chicago [UIC], standard). Each of our 3 ERG laboratories (located in Montreal, Quebec [McGill University], Chicago [UIC], and Rome [Regina Elena Institute]) has its own reference range of ERG values, uses different light intensities, and uses different electrodes. To make meaningful comparisons between the 3 sets of data, we decided to normalize ERG values by expressing them as percentages of the lowest limit of each of our reference ranges.

PSYCHOPHYSICS

To assess sensitivity losses across the retina, we performed static threshold perimetry with 500- and 650-nm stimuli after 1 hour of dark adaptation using a Tubinger perimeter (Oculus, Inc, Tubingen, Federal Republic of Germany) on subjects 1 and 2 of family 1 as previously described.29

RESULTS

We found abnormal ERGs in all 4 subjects from both families, while we did not find any ERG abnormalities in the
12 additional LCA-affected parent pairs who were negative on our GUCY2D, RPE65, CRX, and AIPL-1 genetic screening (data not shown). Of interest are the similarities in the cone ERG abnormalities in the 4 parents.

**ELECTROPHYSIOLOGICAL ASSESSMENTS AND PSYCHOPHYSICS FOR THE PARENTS OF FAMILY 1 (L954P MUTATION IN GUCY2D)**

The 47-year-old mother (subject 1) has mild asthma. She reported difficulties driving at night, and photophobia during the day. Her vision was correctable to 20/30 OD with $-0.50 +0.75 \times 110^\circ$, and 20/25-1 OS with $+0.50 +0.50 \times 110^\circ$. The results of her retinal examination showed no abnormalities. Her ERGs were performed at The Johns Hopkins University (Baltimore, Md) when the patient was 45 and 46 years old (data not shown) and were repeated at UIC when the patient was 47 years old (Figure 2). Results of the ERGs at the 2 institutions were similar. At UIC, we found distinctly reduced 32-Hz flicker and single flash-light–adapted a- and b-wave amplitudes (Figure 2A). The flicker amplitudes were reduced to 137 µV, which represents 80% of the lower limit of normal. The value 230 µV represents the normal average, with a range of 171 to 350 µV in this laboratory. The 32-Hz white flicker responses were significantly delayed to 36 milliseconds (our normal average is 26 milliseconds, with a range of 22.3–31.6 milliseconds). The single flash-light–adapted a- and b-wave amplitudes were also reduced below the normal range, but their implicit times were normal. The a-wave amplitudes were reduced to 60 µV, which represents 86% of the lower limit of normal. Our normal average is 98 µV, with a range of 70 to 138 µV. The b-wave amplitudes were reduced to 126 µV, which represents 95% of the lower limit of normal. Our normal average is 225 µV, with a range of 132 to 320 µV. For the dark-adapted ERG responses, including both the rod isolated and rod dominant waveforms, we found subnormal values, while the implicit times were normal (Figure 2A). Static threshold perimetry (Figure 3) showed normal rod thresholds.

The 55-year-old father (subject 2) reported a recent onset of impaired vision. He denied having nyctalopia and side vision loss. His vision was correctable to 20/20 OD with $-2.50 +0.50 \times 60^\circ$ and 20/20-1 OS with $-2.50 +0.50 \times 100^\circ$. We found a normal retinal appearance. His ERGs were performed at The Johns Hopkins University when he was 53 years old and again when he was 54 years old (data not shown); when he was 55 years old, we repeated ERGs and performed static perimetry thresholds (Figure 2 and Figure 4) at UIC. The results of the ERGs at both institutions were very similar; therefore, we only report the most recent results from UIC. The timing and amplitudes of the rod-mediated ERGs were subnormal (Figure 2A), while the cone-mediated ERGs, especially the 32-Hz flicker were again markedly decreased (Figure 2B). The 32-Hz flicker (Figure 2B) am-
plitudes were decreased to 152 µV, which represents 89% of the lower limit of normal. Our normal average is 230 µV, with a range of 171 to 350 µV. The flicker responses were also slightly delayed to 32 milliseconds, while our normal average is 26 milliseconds, with a range of 22 to 31.6 milliseconds. The single light-adapted response was within the lower limit of normal for the b-wave amplitude with a normal implicit time. The a-wave ampli-
decrease, however, was reduced to 60 µV, which represents 86% of the lower limit of normal. Our normal average is 98 µV, with a range of 70 to 138 µV. Static threshold per-

ELECTROPHYSIOLOGICAL ASSESSMENT FOR THE PARENTS OF FAMILY 2
(P575L MUTATION IN GUCY2D)

The 48-year-old mother (subject 3) has a history of myo-
pia and a lifelong history of photoaversion. She denied hav-
ing difficulties with night vision. Visual acuities were 20/15 with −1.50 +1.00 × 90° OU. Findings from her retinal ex-

Figure 4. Static perimetry testing using 650-nm and 500-nm stimuli on the carrier father (subject 2 of family 1) at the University of Illinois at Chicago. cd indicates candela.

Figure 5. Electroretinogram done in Rome, Italy, with a healthy subject, mother (subject 3), and father (subject 4) of family 2, respectively. A indicates rod isolated; B, maximal rod and cone; C, cone isolated electroretinogram; and D, 30-Hz flicker.
cluster of small hard drusen, temporal to the fovea in each eye. The rod-mediated responses of subject 4 were within normal limits (Figure 5) as were the maximal ERG responses. Light-adapted, cone-driven, transient and 30-Hz flicker responses to the 1.5–cd/s/m^2 stimuli were normal in timing (interocular average: transient, 31 milliseconds; flicker, 32 milliseconds), but again subnormal in amplitude (interocular average: 52 µV for the transient and 36 µV for the flicker response, which correspond to about 70%-75% of the lowest limits of normal). The responses to 3.5–cd/s/m^2 stimuli (not shown) were proportionally larger in amplitude (90.5 µV, which is 88% of the lowest limit of normal) and remained normal in timing (29.5 milliseconds), suggesting a loss in cone-mediated sensitivity.

We performed a functional assessment on heterozygous parents of offspring with LCA who have mutations in GUCY2D and found significant diffuse cone-mediated ERG abnormalities in 4 parents, and subnormal rod isolated amplitudes in 2. To our knowledge, this is the first report of abnormalities of the cone-rod systems in parents of subjects with LCA. We found these abnormalities in 2 sets of parents whose offspring were diagnosed as having LCA and who each carry 1 identified GUCY2D mutation. There were no significant differences in the electrophysiological abnormalities of the 2 families, despite the distinct locations of the mutations, 1 in the catalytic and 1 in the kinase homology domain of the GUCY2D protein. Cone amplitudes were decreased in both parents of both families, while cone-mediated signals were delayed in 2 of the parents (subjects 1 and 2). The electrophysiological changes of the LCA carriers are most consistent with a mild cone-rod dysfunction. Not all carrier parents of children with LCA have abnormal ERGs; we found normal ERGs in 12 parent pairs who were negative for GUCY2D as well as RPE65, CRX, and AIPL-1.

The observed cone ERG abnormalities in these 2 sets of parents are quite similar to one another and these in vivo findings correlate well with our previous in vitro experiments (C. L. Tucker, PhD, V. Ramamurthy, PhD, A. L. Pina, PhD, unpublished data, June 2002). In these experiments, we co-expressed the GUCY2D L954P mutation with the wild type allele and found dose-dependent, dominant-negative effects. As we increased the dose of the mutant, the ability of the wildtype allele to synthesize cGMP under GCAP1 and GCAP2 stimulation was progressively impaired in our HEK293 cell culture system (C. L. Tucker, PhD, V. Ramamurthy, PhD, A. L. Pina, PhD, unpublished data, June 2002). Our cone photoreceptor–mediated abnormalities also correlate well with the known expression profile of GUCY2D in the human retina, which is expressed at considerably higher levels in cone photoreceptor outer segments than in rod outer segments. Using immunocytochemical methods, Liu et al34 and Dizhoor et al35 showed that the cone outer segments were more densely labeled with an antibody to GUCY2D than the rods were. Also, a cone-specific dystrophy was found when the GUCY2D gene was knocked out in the mouse. Yang et al36 showed that GUCY2D knockout mice develop a severe and rapid cone degeneration with cone ERG loss, while the rods remained morphologically normal. There are several limitations to the specificity of our findings that must be noted. Our inability to find the second mutant GUCY2D allele in patients with LCA identified as having one probable disease-associated mutation in a known LCA gene is a relatively frequent finding reported by several other investigators who study LCA genes. For example, in the study by Lotery et al21 of diseases causing mutations in GUCY2D were identified in both alleles in only 2 of 11 patients. This example underscores the challenge posed by identifying both disease-causing mutations in autosomal recessive retinal diseases, for example, those that are located in midintranons and in promoter regions. The possibility that LCA may be caused by a digenic mechanism in our 2 families cannot be excluded, although the likelihood of this is low.

Heterozygous mutations in GUCY2D can cause an early-onset, severe dominant form of cone-rod dystrophy, unlike the clinical phenotype of the 4 heterozygous parents evaluated in this study. While it cannot be excluded that the GUCY2D mutations in our patients exhibited incomplete penetrance or effect modification owing to differences in genetic background between the mothers and their affected offspring, the negative family history suggests that our patients with LCA most likely carry 2 disease-causing GUCY2D mutations in the compound heterozygous state, and that we were only able to identify the recessive mutation on the mother’s allele in both families, but not the mutation on the father’s allele. We also propose that LCA in our patients is the result of 2 recessive mutations (in our cases compound heterozygotes), and in single dose, these heterozygous mutations (L954P and P575L) may cause a mild cone-rod dysfunction.

Electoretinographic abnormalities have been previously reported in parents of children with other recessive retinal degenerations. For example, Rosenfeld et al30 found small rod-mediated abnormalities in carriers of patients with autosomal recessive RP and the rhodopsin genotype. Felius et al31 found extensive dark-adapted visual field defects (1-2 log units above normal) and delayed 30-Hz flicker ERG in 1 parent of a child with LCA who has the RPE65 genotype, while Swaroop et al32 found small rod-cone abnormalities in parents of a child with LCA who has the CRX genotype. Parents of children who have recessive Bardet-Biedl were found to have small cone ERG abnormalities (Elise Heon, MD, oral communication, June 1, 2001) and small rod ERG abnormalities.33 While we have found mainly cone abnormalities in the parents of the child with LCA who has the GUCY2D genotype, ERG changes in parents of children with LCA who have the AIPL-1, TULP1, CRB-1, RGRIP-1, and LRAT genotype have not yet been reported. Whether these novel and subtle ERG changes in parents of patients with LCA are gene specific and can point us to the pathogenic gene must await confirmation after more extensive genotype-phenotype correlations.

We documented the occurrence of predominantly cone ERG abnormalities in heterozygous parents of patients with LCA, likely attributable to the carrier state for GUCY2D mutations. The abnormalities are most consistent with a mild cone-rod dysfunction. Given the sub-
stantial genetic heterogeneity of LCA (10 genes so far), and the possible gene-specific nature of future treatments, rapid genotyping of patients with LCA is essential. It would be helpful if gene-specific ERG changes could be consistently demonstrated in obligate carriers and point to the defective gene of a patient with LCA. The purpose of a future study will be to test the hypothesis that gene-specific changes can be consistently demonstrated by simple ERG testing in carrier parents of patients with LCA. Future findings from such studies might be useful for more focused molecular genetic testing.

Submitted for publication November 6, 2001; final revision received April 26, 2002; accepted May 1, 2002.

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We thank all of the patients and parents involved. We also thank Ana Luisa Pina, PhD, Magdali Loyer, MSc, Chandra Tucker, PhD, Vishy Ramamurthy, PhD, Jim Hurley, PhD, and Debra Derlacki, BS, for their support.

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