PERGAMON

Vision Research 38 (1998) 3293–3297

Vision Research

M- and L-cones in early infancy: III. Comparison of genotypic and phenotypic markers of color vision in infants and adults

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Received 10 July 1997; received in revised form 15 January 1998

Abstract

Genetic analyses were performed on five male children (~ 3 years), two suspect color-normals and three suspects for congenital color vision deficiencies. These classifications were based on visually-evoked potential (VEP) responses to M- and L-cone-isolating stimuli obtained in a previous study when each subject was either 4- or 8-weeks old. The present analyses were performed in a blind study to characterize the genotypes of these subjects. Four male adults with various color vision phenotypes were also tested as a control. DNA was isolated using a non-invasive technique followed by polymerase chain reaction (PCR) amplification and restriction enzyme analysis to examine the genomic DNA of each subject. The genetic analyses confirmed the VEP identification of two color defective infants, and were consistent with the diagnosis of two other infants as color normal. A third infant was predicted by VEP analysis to have a protan defect, but he did not have a gene array typically found in protan observers. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: M- and L-cones; Early infancy; Genotypic and phenotypic markers; Color vision

1. Introduction

In two previous studies [1,2] we have used a double silent-substitution technique to show that both 4- and 8-week-old infants can generate visually-evoked potential (VEP) responses to stimuli that isolate either M- or L-cones. Our stimulus consisted of three primaries, a 570 nm light alternating in square-wave counterphase with a mixture of 540 and 610 nm. To isolate the response of a single receptor class, for example the L-cones, the intensity ratio of the 540/610 nm mixture and the intensity of the 570 nm light were adjusted so that each field produced equal quantal absorptions in the M-cones and rods, respectively. Thus, when presented successively to the same retinal area, the substitution of one field for the other should be silent for M-cones and rods; any response obtained would then be due to the modulation of only the L-cones. The same logic was also used to isolate responses originating in M-cones and rods, as well. Nearly all of the infants tested displayed measurable VEP responses to both M- and L-cone isolation, suggesting that M- and L-cones are functional by 4-weeks postnatal. A subsequent study [3] verified that these responses were indeed mediated by M- or L-cones in that their spectral sensitivities corresponded to the [4] M- and L-cone fundamentals, respectively.

We have also reported data obtained from four male infants, each of whom demonstrated strong responses to only one of the cone-isolating stimuli (either M or L), but displayed little or no response to the other [5]. These results, combined with subsequent verbal reports from their parents of a maternal history of color vision deficiency, led us to suspect that these males might be color defective. Additional support for the diagnosis of one infant (B1) classified as a protanope comes from his mother. Long-wave spectral sensitivity for this female observer fell midway between the protanopic and color-normal spectral sensitivity curves, characteristic of

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PII: S0042-6989(98)00067-4

many protan heterozygotes [6]. The mother of the other protan suspect (B3) did not demonstrate Schmidt's sign. This finding, however, does not necessarily negate the possibility that subject B3 is a protan. There are sufficient uncertainties in the VEP's of infants to warrant further investigation of these infants to evaluate if the VEP classifications are correct. Here we present the results of follow-up genetic analyses performed on three of the suspect color defective infants and two who were classified as probable color-normals by our VEP method.

Previous studies of the molecular genetic basis of normal and defective color vision have identified specific gene array structures that are often associated with certain color vision defects. For example, individuals with single gene arrays in which the gene encodes an L- or M-pigment are likely to be deuteranopic or protanopic, respectively. Arrays with multiple L pigment genes but no M genes are typical of deuteranomaly, and arrays with multiple M genes but no L genes are typical of protanomaly. Arrays with many L genes and relatively few M genes are also typical of deuteranomaly, and arrays with more M than L genes are most common in color normals. Thus, using genetic analyses, we can characterize the structure of individual gene arrays and make predictions about potential color vision phenotypes. If the structure corresponds to an array that is associated with a specific color vision defect, we can classify the most likely color vision phenotype and establish whether or not it agrees with the classification indicated by our VEP results. It should be noted, however, that the genetic basis for other differences among pigments besides spectral peak, for example optical density, that contribute to color vision phenotype are not well understood [7].

2. Methods

2.1. Subjects

Four male adults (a protanope, deuteranope, color-normal and deuteranomal; A1-A4, respectively) and five male children ($\sim$3 years), two suspect protanopes (B1, B3), a suspect deuteranope (B2) and two suspect color-normals (B4, B5) served as subjects. The color vision status of the adult subjects was determined by the Neitz anomaloscope, Farnsworth Panel D-15, and the American Optical pseudoisochromatic plates. The color vision status of the male toddlers was previously determined at either 4- or 8-weeks postnatal using a double-silent substitution technique combined with a VEP paradigm described elsewhere [8].

2.2. DNA extraction

DNA was extracted from cells obtained from a buccal swab [9]. DNA samples were mailed to the Medical College of Wisconsin for genetic analysis.

2.3. Genetic PCR analyses

Two different assays [10] were performed on the isolated DNA, one designed to estimate the total number of pigment genes on the X-chromosome, and the other designed to estimate the ratio of M- to L-pigment genes. Using the results of both of these assays, it is possible to estimate the total number of M- and L-cone pigment genes on the X-chromosome, from which one can make predictions about phenotypic expression in individual observers.

2.3.1. Total X-linked gene count

A 183 base pair (bp) DNA fragment that lies 50 bp upstream of the 1st exon in each gene was amplified using polymerase chain reaction (PCR) and fluorescently tagged primers, followed by gel electrophoresis. This assay yields two distinct bands. The upper band in the gel is from the first gene in the array, while the lower band represents all of the genes in the array. The total number of genes in the array is estimated by quantitatively evaluating the density ratio of the upper and lower bands by fluorescent-image analysis. Additionally, individuals possessing more than one gene often display faint heteroduplex bands.

2.3.2. M:L X-linked gene ratio

The second assay [10] involved PCR amplification of a 300 bp DNA fragment that includes the fifth exon of each pigment gene on the X-chromosome array. Exon 5 of the pigment genes has been shown previously [11–16] to code for most of the spectral difference between the M- and L-cone pigments. There is an Rsa I restriction site in exon 5 from L-pigment genes that is absent from M-pigment genes. The fluorescently end-labeled PCR product was digested with Rsa I, and the cleavage products were electrophoretically separated. The amount of DNA in each band was quantified using fluorescent image analysis, and the ratio of M/L genes was estimated. Heteroduplex bands are observed for subjects possessing both L- and M-pigment genes.

3. Results and discussion

Table 1 presents the results of molecular genetic analysis and the classifications based on both the genotypes and phenotypes for each adult observer. There was a perfect correspondence between the classifications of each observer, with his phenotype as determined

Table 1
Adult summary

Subject	No. genes	M/L ratio	No. L genes	No. M genes	Phenotype predicted from genetics	Observed phenotype (standard tests)
A1	2.77	33.70	0	3	Protan	Protanope
A2	1.02	0.26	1	0	Deuteranope	Deuteranope
A3	3.01	1.78	1	2	Normal	Normal
A3	4.56	0.33	3	1	Deuteranomalous	Deuteranomalous

using standard psychophysical tests. Given that the procedure was successful with the adults, we then performed the analyses on the toddlers.

Fig. 1 shows the results of the first assay used to estimate the number of visual pigment genes on the X-chromosome for all five toddlers. Each observer is represented in duplicate. The top band comes from the first gene in the array, while the bottom band is from all pigment genes present in the array. Faint heteroduplex bands are present for three of the five subjects (B3, B4 and B5). Heteroduplexes are only seen in this assay for arrays with more than one gene. The total number of genes for each observer, as calculated by fluorescent image analysis, is presented at the bottom of the figure. Heteroduplex bands were not observed for subjects B1 and B2, and the banding patterns are consistent with both subjects having a single X-linked visual pigment gene. Quantitative analysis of the fluorescent image verified that both of these infants have only one pigment gene. These results indicate that subjects B1 and B2 are both dichromats.

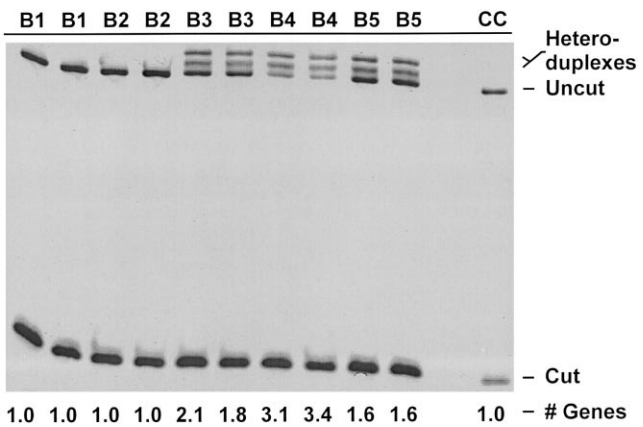

Fig. 1. Total gene count: Results of restriction digestion assay using the Sma 1 restriction enzyme are shown for five toddlers (each presented in duplicate). Two to four bands are present for each subject. For subjects B1 and B2, the upper band represents the first gene in the array, while the lower band is from all genes in the array. For observers B3, B4 and B5, the upper two bands are heteroduplex bands, with the third band coming from only the first gene and the bottom band coming from all genes in the array. The total number of genes calculated for each observer using fluorescent image analysis is shown at the bottom of the figure.

The results of the second assay for subjects B1 and B2 are shown in Fig. 2. Here, the upper band is the uncut fragment that comes from the M-pigment gene, while the two smaller bands below come from the cut L-gene fragments. Note that in order to get enough DNA for this assay, it was necessary to gel purify the PCR product and subject it to a second round of amplification. Because samples from all subjects were gel purified on the same gel, this process resulted in low-level cross-contamination of PCRs from the infants. In Fig. 2, subject B1 has a high density band corresponding to M genes, and extremely faint bands corresponding to L genes. B2 shows fragments from L genes in high density, with an extremely faint M band. Taken together with the results of the assay in Fig. 1 indicating that both B1 and B2 are dichromats, the results from this second assay allowed us to type B1 as

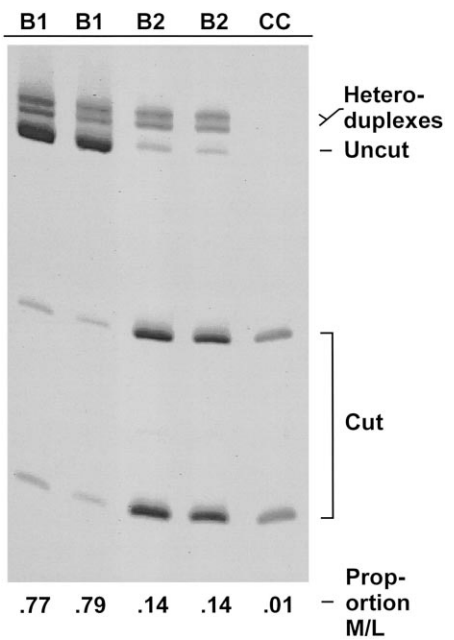

Fig. 2. M:L gene ratio: Results of restriction digestion assay with the Rsa 1 restriction enzyme are shown for observers B1 and B2 (each presented in duplicate). Cut and uncut bands are labeled. The far right lane is a control to insure that the Rsa 1 enzyme is cutting the L-gene fragment. Observer B1 displays the uncut M-gene band in high density, while B2 displays the cut L-gene bands in high density. The relative M:L gene ratio calculated for both observers is shown at the bottom of the figure.

Table 2
Infant summary

Subject	No. genes	M/L ratio	No. L genes	No. M genes	Phenotype predicted from genetics	Phenotype predicted from VEP
B1	1.0	0.78	0.20	0.80	Protan	Protan
B2	1.0	0.07	0.90	0.01	Deutan	Deutan
B3	2.0	1.00	1.16	1.05	Normal	Protan
B4	3.3	3.00	0.80	2.44	Normal	Normal
B5	1.6	1.60	0.60	0.99	Normal	Normal

a protanope, and B2 as a deuteranope, despite the cross-contamination of the infant samples. Table 2 presents a summary of the results from the genetic analyses along with the predicted phenotypes based on the genetic and VEP results, for all five infants. The results for B1 and B2 are consistent with our previous conclusions based on the VEP procedure in that B1 and B2 displayed attenuated signals from L- and M-cone isolating stimuli, respectively. Further support for the classification of B1 as a protanope was provided by the demonstration of Schmidt's sign by his mother.

Subjects B3, B4 and B5 each display genotypes consistent with a normal phenotype. For subjects B4 and B5, our VEP paradigm indicates that they have functioning M- and L-cones given that they show reliable VEP responses to all of our receptor-isolating stimuli. For subject B3, the results from the genetic assays are not consistent with those obtained with the VEP which suggested a protan defect. Combining adults and infants, the agreement between the behavioral and genetic classifications was statistically significant ($\kappa = 0.833$; $P = 0.003$) [17].

In an attempt to further clarify the genotype of infant B3, we have performed additional analyses using blood-derived DNA collected from both B3 and his mother. We used a newly developed genetic assay that is sensitive to protan heterozygotes [18] to determine whether B3's mother has a gene array structure typical of a protan carrier. The results suggest that she does not have the gene structure typical of someone who is heterozygous for a protan defect. This is consistent with the previous observation that B3's mother does not display Schmidt's sign. Furthermore, for subject B3 the same results (a 1:1 M:L gene ratio) were obtained upon repeating the analyses using blood-derived DNA, which incidentally, provides yet another measure of the reliability of these results. An M:L gene ratio of 1:1, typical of individuals with normal color vision [10,11], would presumably rule out protanopia unless he does not express this pigment in his retina. It should be noted, however, that a male with a protan defect possessing both M and L genes has been reported in the literature [11]. Thus, we cannot rule out the possibility that subject B3 has a protan defect.

4. Conclusions

The results from the genetic analyses were perfectly consistent with the color vision phenotype of the four adults tested. Second, the genetic analyses confirmed VEP identification of two infants with abnormal function of at least one cone class and are consistent with the VEP identification of two others for whom cone function seems normal. These results suggest that our receptor-isolation technique is capable of identifying color deficient infants as young as 4-weeks. Finally, these results provide additional support for our conclusion based on our previous VEP work, that both the M- and L-cone photoreceptors are functional as early as 4-weeks of age.

Acknowledgements

We gratefully acknowledge the help of Andrew Smolen and John Hewitt at the University of Colorado, Institute for Behavioral Genetics. This work was supported by NIA grant AG04058, NICHD grant HD19143, NEI grants EY09303, EY09620, EY01931, an RPB James S. Adams Scholar award to MN, and an unrestricted RPB grant to the Department of Ophthalmology MCW.

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