Serine/Alanine Amino Acid Polymorphism of the L-Cone Photopigment Assessed by Dual Rayleigh-Type Color Matches

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The dual Rayleigh-type color match is the ratio of 621 nm light to 550 nm light that in admixture matches 586 nm light, divided by the ratio of 667 nm light to 550 nm light that in admixture matches 556 nm light. Compared to the classical Rayleigh match, the dual-match procedure minimizes variation in color matching arising from differences in lens pigmentation and photopigment optical density, and thus amplifies individual differences due to shifts in λ_{max}. We hypothesized that the dual matches would provide a clearer distinction between subjects with serine and subjects with alanine than would the classical Rayleigh match because individuals with serine express L pigments with a longer wavelengths than those with alanine. Classical Rayleigh color matches were compared with dual Rayleigh-type color matches in 14 color-normal observers whose DNA had been analyzed previously for the presence of the amino acid serine or alanine at position 180 in the L opsin. The resulting distribution of dual-match measurements for the seven subjects with serine does not overlap the distribution of measurements for the seven subjects with alanine. The classical Rayleigh-match measurements for these two groups of subjects, on the other hand, overlap substantially. More than half of the subjects' classical Rayleigh matches are within the overlapping range. The dual Rayleigh-type matches, therefore, provide an improved psychophysical technique for assessing whether an individual observer has serine or alanine at position 180.

INTRODUCTION

Foveal color matches of spectral lights above 545 nm are mediated in normal trichromats by two types of photoreceptors: the middle-wavelength-sensitive (M) and long-wavelength-sensitive (L) cones. A physical admixture of two primary lights (say, 545 and 670 nm) can be set to match any spectral light in this range. Typically the admixture and the spectral light are viewed side-by-side in a bipartite circular field. The observer adjusts (i) the proportion of long-wavelength light (670 nm) in the admixture and (ii) the radiance of the spectral light. The admixture matches the spectral light when the rate of quanta1 absorption by the M cones is identical in each hemifield and, simultaneously, the rate of quanta1 absorption by the L cones is the same in each hemifield (Pokorny, Smith, Verriest & Pinckers, 1979; Wyszecki & Stiles, 1982).

The rate of quanta1 absorption by a cone pigment illuminated by a given physical light depends on the photopigment's wavelength of peak sensitivity (λ_{max}). All other things equal, if two observers do not differ in the λ_{max} wavelengths of their M and L photopigments then this pigment difference will be reflected in their distinct color matches. Systematic variation in the λ_{max} of the L cone pigment is known to be caused by an amino acid polymorphism of the L opsin (Neitz, Neitz & Jacobs, 1991; Winderickx, Lindsey, Sanocki, Teller, Motulsky & Deeb, 1992b; Merbs & Nathans, 1992). The substitution of the hydroxyl-bearing amino acid, serine, by the non-polar amino acid, alanine, at position 180 in the L opsin has been found to correlate with differences in a color match of 551 + 667 nm primaries to 590 nm light (Winderickx et al., 1992b). This color match is very similar to the classical Rayleigh equation. The correspondence, however, between the Rayleigh-match value and the amino acid at position 180 (serine or alanine) is only a statistical
relation. While the Rayleigh-match midpoint is lower on average for observers with serine, the range of midpoint values for observers with serine overlaps substantially the range of values for observers with alanine. The midpoint measurements of 44% of the individuals studied by Winderickx et al. (1992b) fall in the overlapping region (defined by the smallest midpoint value for an observer with alanine and the largest midpoint value for an observer with serine). The classical Rayleigh match, therefore, is an imperfect indicator of whether a particular individual has serine or alanine at position 180.

The broad distribution of Rayleigh-match values among individuals with identical wavelengths of $\lambda_{\text{max}}$ is not unexpected. A difference in $\lambda_{\text{max}}$ is only one of the physiological sources of variability in classical Rayleigh matches. Rayleigh-match measurements are affected also by pre-receptoral spectrally selective filtering, such as that caused by the lens, and by the optical density of photopigment in the outer segment of receptors. Normal variation in pre-receptoral filtering and optical density can alter the value of the classical Rayleigh match by an amount similar to that caused by a $\pm 5$ nm shift in $\lambda_{\text{max}}$ (He & Shevell, 1993). Quantitative estimates suggest that the shift in $\lambda_{\text{max}}$ of the L pigment when serine is substituted by alanine in the L opsin is 3-5 nm toward shorter wavelengths, based on both color matching and spectrophotometric measurements of pigments expressed in vitro (Neitz & Jacobs, 1986, 1990; Merbs & Nathans, 1992; Sanocki, Lindsey, Winderickx, Teller, Deeb & Motulsky, 1993). The Rayleigh-match measurement, therefore, is an index that is affected by multiple factors and thus cannot isolate a shift in photopigment $\lambda_{\text{max}}$ caused by the substitution of serine by alanine. While a large sample of classical Rayleigh matches is useful for estimating the population distribution of measurements for observers with serine or alanine, from which the mean of each distribution can be estimated and related to a difference in $\lambda_{\text{max}}$, the classical Rayleigh match is an unreliable index of the photopigment in the eye of an individual observer.

An alternative color-matching procedure can potentially distinguish a shift in the $\lambda_{\text{max}}$ of the L pigment from other factors that contribute to variation in the classical Rayleigh match (Neitz & Jacobs, 1990; He & Shevell, 1993). The procedure, referred to as dual Rayleigh-type color matches, compares two color matches in ratio. One match is similar to the classical Rayleigh equation, and the other match uses a different long-wavelength primary (621 nm in place of 667 nm). The ratio of these matches is more sensitive to a small difference in the $\lambda_{\text{max}}$ of the L pigment than to variation in lens transmissivity and optical density (see He & Shevell, 1993, for a detailed analysis). Because the dual-match ratio amplifies the effect of small differences in L pigment $\lambda_{\text{max}}$ relative to the effects of pre-receptoral filtering and photopigment optical density, this procedure may be a behavioral measure of the expressed photopigment (phenotype) that is useful for assessing gene polymorphisms, such as that resulting in the substitution of serine by alanine (genotype).

The purpose of the present paper is to extend the results of He and Shevell (1993) to include subjects whose DNA has been analyzed previously for L opsin gene amino acid polymorphisms. Dual Rayleigh-type color matches are compared with classical Rayleigh matches from color-normal subjects known to have either serine or alanine at position 180 in the L opsin gene. We show that the dual Rayleigh-type color matches discriminate subjects with serine from subjects with alanine much more clearly than does the classical Rayleigh match. In our sample, the dual Rayleigh-type match value has a distribution for observers with serine that does not overlap the distribution for observers with alanine. By comparison, the classical Rayleigh-match measurements for more than 50% of these same observers fall in an overlapping region. Thus, the dual-match ratio is more strongly associated with the amino acid found at position 180 than is the classical Rayleigh match.

METHODS

Subjects

Sixteen color-normal Caucasian males, aged 19-24 yr, were selected for the study. The subjects were randomly chosen for recruitment from a group of 48 color-normal Caucasian males having a polymorphism at position 180 in the L opsin, as reported by Winderickx et al. (1992b) and Sanocki et al. (1993) (but see below for a brief summary of the molecular analyses used in the previous studies). All subjects were close in age and were emmetropic (20/20 visual acuity). This enhanced the homogeneity of the group. All subjects' opsin genes had been sequenced entirely, and with the exception of one subject (subject No. 8), the amino acid polymorphism at position 180 was the only non-homologous amino acid substitution found in each subject's L opsin. Subject No. 8 had non-homologous amino acid substitutions at position 230, where isoleucine was substituted by threonine, and at position 233, where alanine was substituted by serine. For the M opsin, the amino acid alanine was found at position 180 in all the subjects' genes. One subject (subject No. 1), with serine in his L opsin, had both serine and alanine in his M opsin.

Molecular analysis

For each subject, the coding sequences of the exons constituting the L and M opsin genes had been determined previously using a combination of polymerase chain reaction (PCR) amplification, sequencing and single stranded conformation polymorphism (SSCP) analysis, [these are described in Winderickx, Battisti, Hibiya, Motulsky and Deeb (1994); and summarized extensively in Sanocki et al. (1993)]. The following is a brief description of these molecular analyses.

Each subject's DNA was first screened for the presence of hybrid genes using Southern blot analysis of DNA restriction fragments, as described by Deeb, Lindsey, Hibiya, Sanocki, Winderickx, Teller and Motulsky (1992). As the presence of hybrid genes may complicate
interpretation of SSCP analysis, subjects showing evidence of hybrid genes were eliminated from further analyses.

Next, the exons of the L and M opsin genes were amplified in two rounds of PCR and were analyzed using SSCP. Exons were amplified using each of two strategies; one that employed gene-specific primers and another that used common primers (i.e. primers that recognize sequences in both the L and M opsin genes). The sequence of these primers have been published previously (Winderickx et al., 1992b; Winderickx, Sanocki, Lindsey, Teller, Motulsky & Deeb, 1992c; Winderickx, Battisti, Motulsky & Deeb, 1992a; Sanocki et al., 1993; Winderickx et al., 1994).

To determine the amino acid present at position 180 of the L or M opsins, L and M exon 3 sequences were amplified in the first round of PCR using common primers and gene-specific primers. Further, gene-specific amplification of exon 3 was initiated in separate reactions from different locations in the gene using primers that were specific for sequences found either upstream (5') or downstream (3') from exon 3.

The fragments obtained in the first-round PCR reactions were then purified and used as templates in second-round PCR reactions to amplify exon 3 for sequence or SSCP analysis. The second-round of PCR reactions were performed using common L and M primers in exon 3.

Finally, the DNA fragments were analyzed by SSCP. Amplification products that yielded unique migration patterns from different locations in the gene using primers that were specific for sequences found either upstream (5') or downstream (3') from exon 3.

The reliability of an observer's matches is assessed by comparing the independent measurements taken on each eye.

Stimulus presentation was computer-controlled and followed an interleaved double staircase procedure that randomly inserted unrecorded trials to prevent subjects from detecting a pattern of stimulus presentation. The 1-sec:1-sec light–dark cycle was used to optimize color discrimination (cf. Nagy, 1980). One staircase determined the lower end of the match range, while the other determined the upper end of the range. Each end point was estimated by the average of six reversals recorded upon reaching the criterial step size. The midpoint of the matching range was taken as the midpoint measurement.

Each subject's color matches were converted from the proportion of the red primary in the mixture field at the match, "R/(R + G)" ["I_{621}/(I_{621} + I_{550})" or "I_{621}/(I_{621} + I_{550})"], to the ratio "R/G" ["I_{621}/I_{550}" or "I_{621}/I_{550}"], and were then scaled to be expressed in deutan mode (Pokorny, Smith & Katz, 1973). For the classical Rayleigh matches, the deutan-mode "I_{621}/I_{550}" value was re-expressed as "I_{621}/(I_{621} + I_{550})". For the dual matches, the ratio K was calculated as the deutan-mode ratio "I_{621}/I_{550}", from the match with the 621 nm primary, divided by the deutan-mode ratio "I_{621}/I_{550}", from the match with the 667 nm primary.

RESULTS

The observers participated in only session so reliability of measurement was assessed by comparing results for each subject's left eye and right eye. Two of the 16 observers (one with serine and the other with alanine at position 180 in the L opsin) had large discrepancies between their two eyes and therefore were dropped from further analysis. It is virtually certain that the interocular difference is due to measurement error of one of the four matches, based on other color matches from these subjects. Unfortunately, the subjects could not be recalled for further measurements.

Classical 2 deg Rayleigh match midpoints using the 667 nm primary in the mixture field are shown in Fig. 1 for 14 observers. The subjects with serine at position 180 on their L opsins are designated by open symbols and those with alanine by solid symbols. Results for the right and left eyes, measured on separate runs, are shown as
squares and circles, respectively. The results of the matches from the subject with alanine who also had positions 230 and 233 amino acid substitutions, are shown for the right and left eyes as an x and a diamond, respectively. The proportion of 667 nm light in the match \( \frac{I_{667}}{I_{667} + I_{550}} \) is shown on the abscissa, and the subject number is shown on the ordinate. The theoretical value of the match midpoint for an observer with L and M cone spectral sensitivities of the standard-normal observer (He & Shevell, 1993) is shown by an arrow on the horizontal axis. Individual differences in the midpoints are apparent, as found by many other investigators and discussed in the introduction.

The distribution of midpoints from the subjects with serine is significantly different from the distribution from subjects with alanine (two-tailed t-test, \( P < 0.025, \) data averaged over both eyes). These results replicate those reported by Winderickx et al. (1992b) and Sanocki et al. (1993) on the same subjects, by showing that the Rayleigh match midpoints of subjects with serine at position 180 in their L opsins are significantly different from those of subjects with alanine at the same position. The subjects with alanine tend to have midpoints displaced farther toward the 667 nm primary, compared to the subjects with serine, indicating that the subjects with alanine are less sensitive to the 667 nm light. There is considerable overlap, however, of the distributions of midpoints for the subjects with serine and with alanine, thus preventing a clear distinction between those subjects with serine and those with alanine. This is shown in Fig. 1 by the shaded region, which contains the measurements of nine of the 14 observers.

The results of the dual Rayleigh-type color matches are shown in Fig. 2 for the same subjects. Those with serine are indicated by open symbols and those with alanine by solid symbols. Results for the right eye and left eye, measured on separate runs, are shown as squares and circles, respectively. The results of the matches from the subject with alanine who also had positions 230 and 233 amino acid substitutions, are again shown as an x and a diamond for the right and left eyes, respectively. The ordinate shows the subject number and the abscissa shows the value \( K = \frac{I_{667}}{I_{667} + I_{550}} \). The arrow on the horizontal axis is the theoretical \( K \) value for a standard-normal observer. The vertical line divides the \( K \) values for subjects with serine in the L pigment from those with alanine. \( K \) values, averaged for both eyes were 1.366 and 1.181 for subjects with serine and with alanine (excluding subject No. 8), respectively.

As was the case with the classical Rayleigh matches shown in Fig. 1, the distribution of dual match values for the subjects with serine is significantly different from that for the subjects with alanine (two-tailed t-test, \( P < 0.0001, \) data averaged over both eyes). In contrast to the classical Rayleigh results (Fig. 1), however, the \( K \) values from the subjects with serine are separated from the \( K \) values from the subjects with alanine (Fig. 2). The dual Rayleigh-type match values form non-overlapping distributions.
Among the subjects with alanine at position 180 in their L opsins, subject No. 8 has additional amino acid substitutions at positions 230 and 233, and has a K value that is displaced furthest from the distribution of dual match values for the subjects with serine. The displacement is consistent with the results of Winderickx et al. (1992b) where the substitutions at positions 230 and 233 appear to affect spectral tuning independently of that seen when serine is exchanged for alanine at position 180. Like the substitution at position 180, the positions 230 and 233 substitutions appear to shift the $\lambda_{\text{max}}$ of the L pigment toward shorter wavelengths.

Finally, among the subjects with serine at position 180 in the L opsin, subject No. 1 had both serine and alanine at position 180 in his M opsins, while all other subjects had alanine only. The dual match is less sensitive to shifts in the $\lambda_{\text{max}}$ of the M pigment when compared to those for the L pigment. However, the M opsin with serine either had negligible influence on his dual match value, or was not expressed in his retina. The dual match value for subject No. 1 was the closest, among those subjects with serine at position 180 in the L opsin, to the distribution of dual match values for the subjects with alanine at the same position. If serine in the M opsin influenced his dual matches, then his K value should have been farther from the alanine distribution, rather than closer. Testing for expression currently is not possible for intact retinas, so the question of expression remains unanswered.

**DISCUSSION**

A comparison of classical Rayleigh matches with dual Rayleigh-type color matches in the same group of subjects shows that the dual Rayleigh-type color matches separate more clearly subjects who differ with respect to the amino acid at position 180 on their L opsins (serine or alanine). Compared to the classical Rayleigh match, the dual Rayleigh-type color matches are more sensitive to small differences in the $\lambda_{\text{max}}$ of the L pigment than to variation in lens transmissivity and optical density (He & Shevell, 1993). As the substitution of serine by alanine is associated with a shift in the L pigment $\lambda_{\text{max}}$ toward shorter wavelengths (Neitz et al., 1991; Merbs & Nathans, 1992; Sanocki et al., 1993), the ratio of dual matches K is better suited for assessing whether serine or alanine is expressed by an observer’s L opsin gene.

The classical Rayleigh match is less useful as an indicator of the L pigment $\lambda_{\text{max}}$ because classical Rayleigh matches are about as sensitive to individual differences in lens transmissivity or optical density as to small changes in L pigment $\lambda_{\text{max}}$. Thus the classical Rayleigh match is an unreliable index of the photopigment in the eye of an individual observer. The dual Rayleigh-type color matches, on the other hand, provide a behavioural measure more closely associated with a genetic variation expressed phenotypically as a shift in L pigment $\lambda_{\text{max}}$.

A minor drawback of the dual-matches compared to the classical Rayleigh match is that K, which is a ratio of two matches, is more sensitive to measurement error. We assessed reliability of measurement here by determining K independently in the left eye and right eye of each observer, a procedure we recommend if a subject will be tested in only one session. Each color match can be completed in <10 min. Matches using the left eye and right eye of a single subject normally are similar (Humanski & Shevell, 1991). A discrepancy between the two eyes indicates that the K values need to be remeasured but does not lead to an inaccurate assessment of the observer’s L pigment. Measurement reliability can be assessed directly, of course, when K is replicated on two or more occasions. The standard error of the mean of repeated measurements usually is small (He & Shevell, 1993).

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