Rate of Flow of Aqueous Humor Determined from Measurements of Aqueous Flare

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Measurement of light scattered in the anterior chamber can provide important clinical information about the eye. In this study, a scanning ocular spectrofluorophotometer was used to measure scattering in the anterior chamber. Pathologic scattering (flare) was induced by argon laser photocoagulation of the iris in pigmented rabbits. The spectrally corrected intensity of the flare in inflamed eyes decreased as the inverse 2.2 power of the wavelength of the illuminating beam. The strongest signal with this instrument was measured at 470 nm. Diurnal variation of normal and spontaneous scatter from the aqueous humor was measured in nine pigmented rabbits entrained to a 12-hr light/12-hr dark cycle and in eight human subjects. A technique is described for determining the rate of flow of aqueous humor from scattered light when the transfer rate of scattering substance into the anterior chamber is constant. This technique was used to study changes in flow over the diurnal cycle. The rate of flow in human subjects during sleep was 60% of the rate during waking hours. In rabbits, the rate was lowest (44% of maximum) during the 6 hr after the lights were turned on, and were greatest just before the lights were turned on. These findings confirm previous studies of the circadian rhythm of aqueous humor flow in rabbits and humans. Invest Ophthalmol Vis Sci 31:339–346, 1990

Materials and Methods

Measurement of Flare

A scanning ocular spectrofluorophotometer (SOSF), developed in our laboratory, was used to measure scattered light. The wavelength of the excitation monochromator was varied, according to the beam and measuring the amplitude of the modulated signal. Sawa et al recently described an instrument based on a similar principle; this instrument measured light scattered from the beam of a He–Ne laser as the beam was modulated back and forth across the measurement window.

Scattering in normal aqueous humor is not constant; it varies over a 24-hr cycle, as does intraocular pressure. Several studies, in rabbits and in humans, have shown that the flow of aqueous humor also varies diurnally. The direction and timing of the variations of scatter and intraocular pressure are consistent with the hypothesis that both are determined by the flow rate of aqueous humor, which in the rabbit is greatest at night and in the human is greatest in the day.

In this study a spectrofluorophotometer was used to measure flare in the inflamed eyes of rabbits and to measure the scattering of light in the normal eyes of rabbits and humans. In the normal eyes, diurnal variations of aqueous humor flow were calculated from these measurements.
experiment, and the emission monochromator was always set to the same wavelength as the excitation monochromator. The power of the light source was reduced by a factor of 10, with a neutral density filter or a reduced aperture on the excitation lens to prevent overload of the photomultiplier tube by the intense scatter from the cornea.

The instrument was operated in two modes, a "fixed-wavelength mode" and a "spectral mode." In the fixed-wavelength mode, the focal point of the instrument was scanned repeatedly through the cornea and anterior chamber without changing the wavelengths of the monochromators. Typically, a group of 12 scans was made over a period of 12 sec, each scan measuring scattered light along the central axis of the eye from the cornea into the first 1-2 mm of the crystalline lens. Most fixed wavelength measurements were made at 470 nm, which gave the strongest signal. In the spectral mode the excitation and emission monochromators were set to identical wavelengths and were simultaneously advanced between each axial scan.

Selection and Correction of Scatter from Anterior Chamber

Individual anterior-posterior scans were graphed on a computer terminal. Scatter from the anterior chamber was selected by positioning a pair of cursors around the region corresponding to the anterior chamber, between the prominent scatter from the cornea and crystalline lens. Typically, two to five points in this region were selected and averaged. In the fixed-wavelength mode, 10-12 scans were aligned on the corneal signal and averaged prior to selecting the scatter from the anterior chamber.

The spectral sensitivity of the instrument was corrected by using measurements of the excitation light scattered from a film of magnesium oxide that had been precipitated onto an aluminum block from a burning magnesium wire (Baker & Adamson Products, Morristown, NJ). Magnesium oxide is known to scatter 97.5-99.5% of incident light at wavelengths between 400 nm and 700 nm.18 The spectrum obtained from the magnesium oxide was used to generate a set of correction factors, one for each wavelength, that eliminated the wavelength dependence of the instrument. Unless otherwise stated, spectra reported in this paper are corrected spectra.

When scattered light is measured in the anterior chamber, some of the light from the excitation beam is scattered as it goes through the cornea. The light scattered out of the beam path can undergo multiple reflections in the cornea and eventually be directed into the acceptance pathway of the photomultiplier tube. Since the wavelength of measurement is the same as the wavelength of excitation, the stray light is added to the scatter signal that originates in the anterior chamber; this stray light can be significant if scatter from the anterior chamber is low, as in an uninflamed eye. The contribution of this stray light to the scatter signal in uninflamed eyes was estimated by measuring the apparent scatter, with the excitation beam displaced to just outside of the measurement window of the photomultiplier tube by interposing a small prism into the beam path (Fig. 1). In a sequence of 12 scans, 6 were made with the beam aligned and 6 with the beam displaced, alternating 2 scans aligned with 2 displaced. The signal in the normal anterior chamber measured with the beam displaced was typically 60% of the scatter with the beam aligned; the difference was considered to be the true scatter from the aqueous humor. This technique is similar to those used by others to eliminate stray light from the signal.3,4

Flare after an Acute Lesion

The time course of flare after an acute lesion was measured in pigmented rabbits. Ten to 20 burns were made with an argon laser photocoagulator (System 920; Coherent, Palo Alto, CA) in one eye of six adult pigmented rabbits. The photocoagulator was set to a 200-μm-spot diameter, 200 mW power, and a 200-msec duration for each exposure. Prior to treatment, each animal was placed in a cloth restraining bag, and proparacaine HCl was applied topically to both eyes.
Measurements of flare were made in the fixed-wavelength mode at 470 nm and in the spectral mode from 400 nm to 700 nm in steps of 10 nm. Scattered light was measured from both eyes for 6–8 hr after the lesion. Measured scatter was not corrected for stray light in these eyes. All experiments were conducted in accordance with the ARVO Resolution on the Use of Animals in Research.

Diurnal Measurements

Scatter from aqueous humor was measured in eight normal male human subjects between the ages of 19 and 22 every 4 hr for 24 hours, starting at 10:00 AM. Subjects slept between 11:00 PM and 6:00 AM in a dark, quiet room close to the fluorophotometry room. During waking hours, the subjects were instructed to go about their normal business. The appropriate informed consent was obtained from each subject prior to study.

Scatter was measured also in nine pigmented rabbits every 4 hr for 24 hr, starting at 8:00 AM. In three of these rabbits, measurements were continued every 4 hr over a period of 80 hr and then repeated at 96 hr, 100 hr, and 104 hr. Overhead fluorescent lights in the animal care facility were automatically turned on at 6:00 AM and off at 6:00 PM. Animals had been trained to this 12-hr light/12-hr dark cycle for at least 1 week prior to measurement. Animals were removed from their cages and placed in a canvas bag during the measurement. No provision was made to keep the animals in total darkness during the measurement or during transport between their cages and the fluorophotometry room, located one floor below. Graphs of scatter were made as a function of circadian time (CT), which starts at 00:00 when the lights were turned on (06:00 AM standard time). Lights were turned off at 12:00 CT.

Calculation of Rate of Flow from Scatter

If the rate of entry of the scattering substance (albumin or other macromolecules) into the aqueous humor is constant and the volume of the anterior chamber is constant, then the changes in scattering occur as the flow of aqueous humor changes. If the scattering substance leaves the eye solely by bulk outflow, then the rate of change of the scattering substance is determined by the rate at which it enters the anterior chamber, minus the rate of loss by bulk flow:

\[
\frac{dM_s}{dt} = Q - M_s k_o
\]

where \(M_s\) is the mass of scattering substances in the anterior chamber, \(Q\) is the rate of entry from the plasma to the aqueous humor (the transfer rate), and \(K_o\) is the loss coefficient from the anterior chamber. Since \(M_s\) is the product of the anterior chamber volume, \(v_s\), and the concentration of the scattering substance, \(C_s\),

\[
\frac{dC_s}{dt} = Q - C_s k_o
\]

or, solving for flow, \(F\),

\[
F = v_s k_o = \frac{Q}{C_s} - \frac{v_s}{C_s} \frac{dC_s}{dt}
\]

This expression is equivalent to the expression for flow used by Krakau. Equation 3 can be approximated by a difference equation when discrete measurements are made:

\[
F_i = \frac{2}{C_{i-1} + C_i} \left( Q - v_s \frac{\Delta C}{\Delta t} \right)
\]

where the subscripts \(i\) and \(i - 1\) refer to the index of the measurement, \(\Delta C = C_i - C_{i-1}\), and \(\Delta t = t_i - t_{i-1}\).

The average flow rate on the time interval \(t_{i-1}\) to \(t_i\), can be calculated by using Equation 4 if \(Q\) is known and if \(C\) is measured at the beginning and end of the interval. The quantity \(Q\) can be determined if flow is measured independently during a sequence of scatter measurements. \(Q\) can be determined also if, for a period of time, the rate of flow is constant and known. Under these conditions, the term \(\frac{\Delta C}{\Delta t}\) is equal to 0, and Equation 4 is reduced to:

\[
F = \frac{Q}{C_o}
\]

or,

\[
Q = F_o \cdot C_o
\]

where \(F_o\) is the rate of flow and \(C_o\) is the concentration of scattering substances when \(\frac{\Delta C}{\Delta t}\) = 0. The rate of entry of macromolecules into the aqueous humor can be determined directly from Equation 6 if the relation between concentration and scattering is known.

For purposes of this experiment, flow was not measured directly in individuals, but was assumed to be 2.5 \(\mu l/min\) over a time interval when flow was maximum (ie, when measured scatter was least) and when scatter was relatively constant. In human subjects, this interval corresponded to the three flare measurements made between 14:00 and 22:00, and in rabbits to measurements made at 18:00 CT and 22:00 CT. The transfer rate, \(Q\), was assumed to be constant throughout the experiment and was calculated from the scatter and an assumed flow of 2.5 \(\mu l/min\) over these time periods.
Calibration of Scatter in the Eye

In order to determine an absolute entry rate of macromolecules into the aqueous humor, one must know their concentration in the anterior chamber, although absolute concentration is not required to determine flow rate, provided that the scatter is proportional to the concentration.

Since albumin is the primary macromolecule found in aqueous humor, scatter was calibrated in units of albumin concentration. Scatter was measured from serial dilutions of albumin in TC199 culture media (no. M3769; Sigma, St. Louis, MO) in cuvettes. Human serum albumin (no. A1653; Sigma) and rabbit serum albumin (no. A0639; Sigma) were measured at concentrations between 0 and 100 mg/dl.

The relationship between scatter in the anterior chamber and scatter of the same aqueous humor in a cuvette was studied in seven pigmented rabbits (14 eyes). The animals were anesthetized, and scatter was measured from both anterior chambers. A 30-gauge needle then was carefully inserted through the cornea, 1-2 mm from the limbus, into the midanterior chamber, and 100-150 μl of aqueous humor was withdrawn. These samples were placed in a 3-mm × 3-mm cuvette, and scatter was measured. They then were analyzed for total protein with a Coomassie Blue dye binding assay (QuanTtest™; Quantimetrix, Hawthorne, CA).

Results

Calibration

Scatter is shown in Figure 2 (top) as a function of human serum albumin concentration. A line was fitted to these data by the method of least squares analysis and was used to calibrate measurements in human eyes.

Scatter from rabbit albumin solutions and aqueous humor are graphed as a function of total protein concentration in Figure 2 (bottom). Scatter from the aqueous humor was approximately twice as strong as from the commercial purified rabbit albumin containing the same concentration of protein. The least squares line through the scatter measured from aqueous humor was used to calibrate total protein in rabbit eyes.

In many rabbit eyes, scatter measured in vivo was higher than scatter measured from the same aqueous humor in a cuvette (Fig. 3). This result indicates that in some rabbits, the presence of the cornea increased the signal from the aqueous humor despite our attempt to correct for stray light. Since this difference was variable from animal to animal, no allowance was made for it. This means that in some animals albumin concentrations were overestimated.

Flare after an Acute Lesion

During the 1st hr after creating an iris lesion, light scattered in the anterior chamber increased rapidly (Fig. 4). An increase in flare was measurable within 10 min, and within 1-1.5 hr it reached a maximum (Fig. 5). After 2 hr, flare began to decrease, and by 6-8 hr, it had returned to near normal. In the contralateral eye, which received proparacaine HCl only, scatter remained normal.
Flare after laser treatment of the iris varied from animal to animal. In one rabbit, 20 burns were concentrated in a focal region approximately 2 mm in diameter. Flare in front of this region increased very rapidly and remained high. A white clot was visible in front of the lesion and caused intense scattering after about 1 hr and gradually dissolved over the next 6 hr. In another rabbit, an increase in flare was not detectable, even though 10 evenly spaced burns were visible on the surface of the iris. Apparently, the intensity of the laser photocoagulator (160–200 mW) was too low to disrupt the blood–aqueous barrier in this rabbit.

In five animals, scatter was measured as a function of wavelength after laser treatment. The wavelength of maximum intensity of the uncorrected spectra was 470 nm (Fig. 6). When the spectra were corrected for the spectral sensitivity of the instrument, flare decreased continuously between 400 nm and 700 nm.

It has been shown that when the diameters of the molecules that scatter light are less than one-tenth the wavelength of the scattered light, intensity decreases as the inverse fourth power of wavelength. As the molecular size of the substance increases, the exponent decreases. The spectra of scatter from five inflamed eyes were fitted by the method of least squares analysis to the following equation:

\[ I = \frac{k}{\lambda^n} \]

where \( I \) is the intensity of scattered light, \( \lambda \) is the wavelength, and \( k \) and \( n \) are constants. The average value of \( n \) for the five rabbits, measured when flare was greatest, was 2.2 ± 0.30 (mean ± SD; \( n = 5 \)).

**Diurnal Changes in Scatter**

In all human subjects, scattering of light in the anterior chamber varied over the 24 hr of observation (Fig. 7). Scatter was relatively stable from 14:00 to 22:00 but increased at night during sleep. In order to calculate the variations in the rate of aqueous flow using Equation 4, we assumed that the rate was 2.5 \( \mu l/min \) between 14:00 and 22:00, when scatter was not changing. During sleep, between 02:00 and 06:00, the average flow was 1.5 ± 0.2 \( \mu l/min \) (mean...
Fig. 7. Average scatter calibrated in units of equivalent albumin concentration (top) and calculated flow rate of aqueous humor (bottom) in eight human subjects. Error bars indicate standard deviation (n = 8). Flow rate decreased during sleep. In calculating flow, it was assumed that flow rate was equal to 2.5 μl/min between 14:00 and 22:00.

± SD, n = 8, subjects), approximately 60% of the assumed rate in the late afternoon and early evening.

The concentration of human serum albumin equivalent to the average intensity of scatter was 17 ± 3 mg/dl during the day and 28 ± 3 mg/dl during sleep. These values are within the range of albumin concentrations measured by others in human eyes. If all of the scatter were due to albumin, then the average rate of entry of albumin, Q, was estimated to be 0.44 × 10⁻⁶ ± 0.07 × 10⁻⁶ gm/min. Based on an average anterior chamber volume of 238 μl in these subjects and an average plasma albumin concentration of 5 gm/dl, the transfer coefficient of albumin into the anterior chamber (Q/[v·albumin concentration in plasma]) can be calculated to be 3.8 × 10⁻⁵ min⁻¹. This is comparable to 5.0 × 10⁻⁵ min⁻¹ measured by Oshika et al using a similar technique.

Fig. 8. Scatter from the anterior chamber in three pigmented rabbits. Each point represents the average of both eyes. Scatter was highest when the lights were on and lowest in the dark.

Fig. 9. Average scatter calibrated in units of total protein concentration (top) and calculated flow rate (bottom) in nine pigmented rabbits. Error bars indicate standard deviation. Flow rate was greatest in these animals during the 6 hr before the lights were turned on. Flow rate was assumed to be 2.5 μl/min between 18:00 and 22:00 CT.

Scatter varied diurnally in the pigmented rabbits also, in agreement with the work of others. The variation measured in three rabbits over 4 days is illustrated in Figure 8. The average variation measured in nine rabbits over a 24-hr period is illustrated in Figure 9. Unlike human subjects, scatter was greatest at 02:00 CT, just after the lights were turned on, and decreased throughout the day. The lowest rate of aqueous flow occurred between 02:00 and 06:00 CT, and the highest occurred between 18:00 and 22:00 CT. The minimum rate was 1.1 ± 0.7 μl/min, 44% of maximum. The average scatter at 22:00 CT was equivalent to 26 ± 4 mg/dl and at 02:00 CT was equivalent to 73 ± 24 mg/dl total protein. Average concentrations of 25–51 mg/dl in the rabbit have been reported by others. Anjou and Krakau reported a range of protein concentrations of 17–103 mg/dl measured at different times of the day, with the greatest in the morning and lowest in the evening.

Discussion

Measurement of light scattered in the anterior chamber with sensitive and objective instruments can be a valuable clinical test to assess the inflammatory response and the breakdown of the blood–aqueous barrier. This technique can provide quantitative information about the natural course of disease and its response to therapy. Unlike subjective evaluation, a radiometer can be used at a specific wavelength and can distinguish scattering from fluorescence.
Although this technique shows changes in the concentration of scattering substances in the aqueous humor, it is not specific for any one substance. For example, scatter from commercial purified rabbit albumin was approximately one half as intense as scatter from rabbit aqueous humor with the same total protein concentration. This difference could be due to the presence of other macromolecules, if they scatter more light than albumin. It is possible that substances other than albumin also contribute to the scatter measured in humans. Albumin concentration in human aqueous humor calculated on the basis of a calibration curve using human albumin gave values close to (although slightly greater than) albumin measured by others. Scatter from these macromolecules should not affect the calculation of flow, provided that their transfer rate into the aqueous humor is constant.

Accurate measurement of substances that scatter light may also be dependent on the depth of the anterior chamber and the clarity of the cornea. A hazy cornea could increase stray light beyond that measurable with the disaligned beam. In rabbits, the anterior chamber is shallower than in humans, and the boundary function from the cornea may have added to the scatter signal from the anterior chamber. If corneal thickness and anterior chamber depth change diurnally, the resulting error might also change diurnally. Although we did not measure corneal thickness or anterior chamber depth after each measurement, the close agreement between calculated changes in flow and flow rates measured by others suggests that our results were not greatly influenced by these artifacts.

In the current series of experiments, it was not practical to measure scatter from aqueous humor in vivo and in vitro in human subjects. Measurements of protein concentration in the anterior chamber may have been overestimated if the cornea influenced measurements, as it appeared to do in the rabbit. Despite this potential problem, there was close agreement between protein concentration and flow measured here and by others. Measurement of scatter from the aqueous humor to determine aqueous flow rate has some advantages over measuring scatter dilution. The greatest advantage is that the technique does not require the application of a xenobiotic tracer, and problems associated with too much, too little, or uneven distribution of fluorescein in the cornea and anterior chamber are not encountered. Long-term measurements of variations in flow can be performed without concern for the eventual disappearance of the tracer from its depot.

The technique also has some disadvantages. First, the calculation of flow is based on the assumption that the rate of entry of macromolecules into the aqueous humor is constant. If this rate is not constant, conclusions that are drawn may be erroneous. The observation that diurnal changes in aqueous flow rate calculated here with scatter were quite close to changes measured with dye dilution techniques indicates that in normal subjects, the rate of transfer, Q, does not change appreciably over 24 hr. One must be aware of the possibility that subtle changes in Q may take place in diseased eyes or in eyes treated with drugs. Any treatment or drug that has the potential of altering vascular permeability also may change properties of the blood–aqueous barrier.

Second, the technique does not give the absolute rate of flow unless the entry rate of the scattering substance is known. The entry rate, Q, can be determined if one can measure flow by an independent means during part of the experiment. In this experi-
centration of dye in the plasma. Second, the tech-
tniques that make use of intravenous injection of
fluorescein are measured. By establishing Q while fluorescein is
present, one can continue to calculate flow from
scatter measurements long after fluorescein has de-
clined to below measurable levels.

The continuous measurement of the entry rate of
macromolecules by simultaneous measurement of
scatter and fluorescein clearance is noteworthy in it-
self. This technique has significant advantages over
techniques that make use of intravenous injection of
fluorescein for evaluating the blood—aqueous barrier.
First, the technique does not require systemic admin-
istration of a dye or frequent measurements of con-
centration of dye in the plasma. Second, the tech-
nique measures the rate of entry of natural substances
rather than a small synthetic compound such as fluo-
rescein. Third, the measurement can be made con-
tinuously over a long period of time rather than over
the short interval immediately after the systemic ad-
ministration of the tracer. One can easily follow
changes in the blood—aqueous barrier that take place
over a time course of several hours.

The measurement of scatter has the potential for
use in the evaluation of the rate of flow of aqueous
humor without the use of fluorescein; the transfer
rate of protein into the aqueous humor; and the
breakdown of the blood—aqueous barrier. These tech-
niques will provide new means of studying the physi-
ologic behavior of the aqueous circulation.

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