Prolonged Monitoring of the Blood–Aqueous Barrier
With Fluorescein-Labeled Albumin

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Aqueous fluorophotometry has proved to be a useful indicator of changes in the blood–aqueous barrier after surgical, immunologic, or laser manipulations. Previously used fluorescent tracers have been unable to follow rapid changes continuously in the blood–aqueous barrier. Fluorescein–labeled homologous serum albumin, however, provides extremely stable and high plasma levels of fluorescence due to active renal reabsorption with very low levels in the normal aqueous because of its high molecular weight. This feature allows prolonged, continuous, and highly sensitive monitoring of the blood–aqueous barrier before, during, and after a manipulation. The usefulness of this technique is demonstrated in a model that has been well studied with other methods: the response to argon laser iris photocoagulation. Invest Ophthalmol Vis Sci 27:415–418, 1986

Observation of the blood–aqueous barrier (BAB) in response to a variety of ocular manipulations has proved to be a useful measure of induced inflammation.1–5 An increase in the aqueous humor concentration of protein or other substances can be determined by paracentesis, but this method destroys the observed system by causing inflammation. Although aqueous fluorophotometry is noninvasive, it requires a suitable tracer with a level that can be readily determined in the aqueous and plasma. The ratio of aqueous to plasma levels indicates the permeability of the BAB to this tracer, which will increase with inflammation.

Sodium fluorescein is the most commonly used tracer because of its availability, low toxicity, and high fluorescence. However, its small molecular weight (MW) allows it to pass through an undisturbed BAB into the aqueous in substantial amounts. Furthermore, its high rates of elimination and metabolism result in rapidly changing plasma concentrations. These problems can be minimized somewhat by using a standardized dosage and determining aqueous fluorescence after a given interval of time. This procedure is completed before and after a manipulation, with the change interpreted as the effect of the manipulation on the BAB. Because this method requires a complete elimination of fluorescein between measurements, some protocols compare the aqueous fluorophotometric measurements between the treated and fellow untreated eye to control for variations in plasma levels of fluorescence.6,7 This modification must be used cautiously, however, as bilateral BAB reactions to unilateral stimulations have been reported.8

Fluorescein isothiocyanate (FITC)-labeled dextran has a higher MW, with resultant lower levels in the anterior chamber and a slightly longer plasma half-life than sodium fluorescein, but both tracers have plasma kinetics that are too complex to assume a steady-state condition in either plasma or aqueous. Therefore, neither can be used to follow rapid changes in the BAB continuously. FITC-labeled homologous serum albumin, however, provides extremely stable and high plasma levels of fluorescence due to active renal reabsorption and low metabolic turnover while maintaining very low levels in the normal aqueous.

The purpose of this report is to demonstrate the ability of this fluorescent tracer to monitor the BAB continuously in a sensitive and prolonged fashion. A simplified method for the synthesis and purification of the FITC-albumin conjugate is detailed. The usefulness of this tracer is demonstrated in a model that has been well studied using other techniques: the response to argon laser iris photoagulation.1–7,9

Materials and Methods

Preparation of rabbit albumin-FITC conjugate (RA-FITC) is adapted from previous reports.10–13 Rabbit albumin, Fraction V (Sigma Chemical Co.; St. Louis, MO), 500 mg, is dissolved in 5 ml of 0.2 M carbonate–bicarbonate buffer, pH 9.4. To simplify the handling of the fluorochrome and to aid in its dispersion, FITC
juvenated by running several volumes of PBS until it protein loss (35%) to the charcoal in our laboratory. quantities but was cumbersome and resulted in excess exchanging the buffers with ultrafiltration, as described activated charcoal to absorb the unreacted FITC before conjugate. The Celite is removed easily with low-speed column, while the unbound FITC remains on the top. The resultant volume of the effluent is approximately is straightforward and easily mastered. Up to 2 g of added to the albumin, stirred, and allowed to react for approximately 1 hr at room temperature. Shorter incubation times may result in a less fluorescent conjugate. The Celite is removed easily with low-speed centrifugation. The next steps involve the separation of the RA–FITC conjugate from the unreacted FITC and the exchange of carbonate-bicarbonate buffer with a physiologic 0.1% phosphate buffered saline (PBS). Both steps are combined when using a medium-grade Sephadex G-25 (Sigma Chemical Co.) gel filtration column loaded with the PBS.

The visual separation of the two fluorescent bands is striking; the RA–FITC conjugate elutes through the column, while the unbound FITC remains on the top. The resultant volume of the effluent is approximately 50% greater than the volume initially added to the column. The yield of albumin is 97%. The column is rejuvenated by running several volumes of PBS until it is visibly clear of fluorescent material. The technique is straightforward and easily mastered. Up to 2 g of conjugate in 20 ml of buffer have been separated in a 2.5 cm dia × 32 cm burette containing a 24-cm column of Sephadex. Larger volumes may be produced by using larger or multiple columns.

These two steps may also be accomplished by using activated charcoal to absorb the unreacted FITC before exchanging the buffers with ultrafiltration, as described elsewhere. This method may be suitable for larger quantities but was cumbersome and resulted in excess protein loss (35%) to the charcoal in our laboratory.

The resultant conjugate is passed through a 0.22 micron filter (Millipore Corp.; Bedford, MA) for sterilization and stored at 0 to 5°C. The unbound fluorescence is determined by measuring the fluorescence of a small sample before and after passage through an ultrafiltration micropartition system (Amicon MPS-1 YM7, Danvers, MA). Less than 1% unbound fluorescence is considered acceptable. The fluorescence vs concentration curve was linear from 1 to 900 fluorescence equivalents of 1 ng of aqueous sodium fluorescein.

Two groups of six Dutch pigmented rabbits (a pilot and repeat study), weighing 1.5 to 2.5 kg each, were given approximately 125 mg of RA–FITC/kg of body weight by rapid intravenous injection in one posterior auricular vein without adverse reaction. Plasma samples were obtained from the other ear at intervals from 1 hr to 7 days. Plasma levels of fluorescence were determined by centrifuging whole blood in a heparinized capillary tube and withdrawing 20 μl of plasma with a micropipette and diluting this into 1.98 ml of PBS. Plasma albumin concentration can be calculated from this by using the fluorescence curve.

The aqueous fluorophotometry of both eyes were done using a commercially available automated fluorophotometer (Coherent; Palo Alto, CA) with an anterior chamber adapter focused over the pupil, a gate time of 100 msec, and 4 steps per millimeter, at 15, 30, 60, 120, 180 min, and at 24 hr. The curvature holder is used for diluted plasma samples.

The argon laser iris photocoagulation was carried out at 48 to 72 hr after injection of the RA–FITC. The right eye was treated with a single burn to the mid-iris, with 0.5 W of energy, 0.2 sec duration, and a 200-μm spot size. The last .05 sec or so of the burn was often interrupted by the animal's blink. To avoid trauma no lid speculum was used. Because numerous medications including topical anesthetics have an effect on the BAB, no medications were used at any time after injection of the RA–FITC.

These investigations conformed to the ARVO Resolution on the Use of Animals in Research.

Results

The time course of the labelled albumin plasma concentration (in μg/ml) in two groups of six rabbits is shown in Figure 1. Each group received a different batch of RA–FITC. Note that the time period in the figure is more than 14 days. Each curve very closely fits (r² = .98) a two-compartment model with a redistribution phase, when the labelled albumin in the plasma equilibrates with the extravascular albumin over 48 to 72 hr, followed by the
slow metabolic decay with a half-life of 8.1 days and 9.3 days for the pilot and repeat studies, respectively. These results are in agreement with those reported previously.10,12,15

Since the pilot study with six animals demonstrated varying response to argon laser iris photocoagulation according to iris pigmentation (brown irides reacted more than blue), we treated six uniformly pigmented rabbits with brown irides in the repeat study. A bubble was formed at the photocoagulation site and prompt miosis was noted. No conjunctival hyperemia was detectable. The results from the repeat study are shown in Figure 2. They are presented as a ratio of anterior chamber to plasma fluorescence. The aqueous fluorophotometry scans at 30 min clearly demonstrated high fluorescence in the anterior chamber directly anterior to the pupil, which is in agreement with the findings of others that the BAB breakdown is predominantly localized to the ciliary body.1,3 The BAB returned to almost normal by 24 hr (3.4 ± 1.2 × 10⁻³, not shown in figure). The untreated fellow eye showed no change during the period studied.

**Discussion**

The FITC-labeled homologous serum albumin has been shown to be stable in vivo and in vitro.10,12 Its biological properties are virtually identical to native albumin, and the label does not render the conjugate antigenic.12 The animals may receive numerous doses of the conjugate without evidence of anaphylaxis. The active renal resorption is the same as for native albumin and affords the tracer a very long and stable plasma level after the redistribution phase.

Because the plasma fluorescence declines minimally over several hours, it allows the rapid changes in the aqueous fluorophotometry values to be interpreted as reflecting only rapid changes in the BAB. Methods using sodium fluorescein depend on all the BAB and plasma fluorescence changes during the interval between its administration and the aqueous fluorophotometry. They are therefore unable to isolate and characterize the rapidly changing permeability of the BAB. Observations over several days also are possible by correcting the aqueous fluorophotometry for the plasma levels but do not require additional administration of the tracer. The absence of a bilateral effect to a stimulus need not be assumed with this technique; in fact, its presence can be quantified.

The large MW of this tracer paradoxically makes it a sensitive indicator of inflammation. Since little albumin is present in normal aqueous (2:1000 ratio of aqueous to plasma fluorescence), a small influx due to BAB breakdown becomes immediately apparent by comparison. This sensitivity is evident in the 50-fold increase in aqueous fluorophotometry after only a single laser burn, far less than previously described doses,1–4,6,9 and one that clinicians may consider minimal.

During the study period the animals are essentially unmedicated so we are able to eliminate the potentially confounding effects of anesthetics and local manipulations, even trauma from a lid speculum.

Okada and Shimada16 used RA–FITC in a model for allergic inflammation, which differs from our report on several points. They did not wait long enough for the plasma levels to stabilize or long enough for the baseline amount of fluorescent protein to enter the aqueous. The amount of inflammation caused by their reverse passive Arthus reaction is unfamiliar compared with that of argon laser iris photocoagulation.

The major drawback of our technique is the current lack of commercially available RA–FITC. Attempts to substitute commercially available FITC-labeled bovine serum albumin caused anaphylactic shock. Fortunately, the methods described above can be carried out with commonly available laboratory equipment in several hours.

The emphasis of this work is not the reaction to iris photocoagulation, which has been reported previously, but rather than unique way in which it was demonstrated. To our knowledge, no other technique has the ability to measure repeatedly the BAB bilaterally in a rapid sequence with such sensitivity over a prolonged period of time in an unmedicated animal.

**Key words:** blood–aqueous barrier, aqueous fluorophotometry, FITC, FITC-labelled albumin, argon laser iris photocoagulation
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References