Sensitive Blood–Retinal Barrier Breakdown Quantitation Using Evans Blue

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Purpose. This study investigated whether a nonradioactive dye, Evans blue, can be adapted as a safe alternative to the isotope-dilution method for quantitating blood–retinal barrier breakdown.

Methods. Blood–retinal barrier breakdown was induced in rats with vascular endothelial growth factor (VEGF) or through the induction of diabetes. After allowing Evans blue to circulate in the vasculature, the dye was cleared from the bloodstream with saline, citrate, or citrate-buffered paraformaldehyde, and the efficacies of the perfusion solutions were compared. Extravasated dye was detected at 620 nm and was normalized against the time-averaged Evans blue plasma concentration, the circulation time, and also against wet and dry retina weights.

Results. Evans blue leakage from retinas treated with VEGF was 4.0-fold higher than that of contralateral untreated eyes (n = 6 rats, P < 0.05). Retinal Evans blue leakage of eyes from 1-week diabetic animals (n = 11 retinas) was 1.7-fold higher (P < 0.05) than that of nondiabetic controls (n = 10 retinas). Intra-animal, inter-retina weights showed significantly less variability (P < 0.05) with the use of dry weights (11.2%, n = 74 retina pairs) than with wet weights (20.5%, n = 95 retina pairs).

Conclusions. The Evans blue dye technique can be modified to be as sensitive and quantitative as the isotope-dilution method for measuring blood–retinal barrier breakdown. The advantages of the Evans blue technique are its safety, relative simplicity, and economy. (Invest Ophthalmol Vis Sci. 2001;42: 789–794)

Blood–retinal barrier breakdown is a key feature of background diabetic retinopathy and a leading cause of vision loss. The in vivo quantitation of blood–retinal barrier breakdown is central to the understanding of retinal vascular disease, as well as to the development of pharmacological therapies. Although animal models manifesting blood–retinal barrier breakdown exist, the quantitation of this phenomenon remains difficult. The powerful isotope-dilution method, using 125I- and 131I-labeled albumin tracers, can sensitively quantitate the blood–retinal barrier breakdown in diabetic animals1–3 or in response to vascular endothelial growth factor (VEGF).4 The isotope-dilution method, however, is relatively expensive and requires the use of highly radioactive isotopes. The present study sought to determine whether Evans blue dye, a widely used nonradioactive intravascular tracer, can be used as an effective alternative for blood–retinal barrier breakdown quantitation.

Evans blue, a tetrasodium diazo salt (MW 980 Daltons), irreversibly5 binds to plasma albumin in a 10:1 molar ratio6 both in vivo7,8 and in vitro.9 In quantitative studies of vascular permeability, Evans blue is injected into the bloodstream, where it rapidly binds to plasma albumin. Whenever plasma extravasates from blood vessels, as with induced vascular permeability, the Evans blue dye–albumin complex leaks into the surrounding tissues. After variable circulation times, animals are then perfused with either citrate-buffered paraformaldehyde10,11 or saline12 to clear the Evans blue from the bloodstream. The extravasated Evans blue can then be extracted from tissues by different solvents, including formamide,11,13 sodium suramin,10 sodium sulfate and acetone,6 and trichloroacetic acid.14 The concentration of dye is then measured by routine spectrophotometry11,15 or florescence spectrophotometry.12 These properties have made Evans blue suitable for the quantitation of plasma albumin leakage secondary to increased vascular permeability in skin,16,17 airways,18,19 brain,14 conjunctiva,20,21 and aqueous humor.22,23 The dye has also been used histologically to localize plasma albumin extravasation in the skin,24,25 conjunctiva,13 ciliary body,26 trachea,12 choroid,12 and retina.5,27–29

Until now, Evans blue quantitation of blood–retinal barrier breakdown, such as occurs in diabetic retinopathy and multiple other eye diseases, has been insufficiently sensitive. In the present study, VEGF was used to rapidly induce extensive breakdown of the blood–retinal barrier, and the ability of a modified Evans blue dye technique to quantitate this breakdown was assessed and compared with historical data using the isotope-dilution method. It was then investigated whether the Evans blue technique might also be sensitive enough to quantitate the less robust blood–retinal barrier breakdown of 1-week diabetic rats. It was also investigated whether the clearance of Evans blue from the plasma of 1-week diabetic animals and age-matched nondiabetic controls is similar. As part of these experiments, various parameters were investigated to optimize the Evans blue technique. They include (1) perfusion with saline, citrate, and citrate-buffered paraformaldehyde to determine which solution is superior for retinal dye recovery, and (2) leakage normalization against wet and dry retina weights. It was hypothesized that Evans blue dye, if suitably adapted, might serve as an effective alternative to the isotope-dilution method for quantitating blood–retinal barrier breakdown.

Materials and Methods

Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA), weighing approximately 200 g, were used in this study. The animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental procedures used aseptic sterile techniques and were approved by the Animal Care and Use Committee of the Children’s Hospital, Boston, Massachusetts.
Evans blue dye (Sigma, St. Louis, MO) was prepared by dissolving it in normal saline (30 mg/ml), sonicating it for 5 minutes in an ultrasonic cleaner (G1125P1T; Laboratory Supplies, Hicksville, NY), and filtering it through a 5-μm filter (Millipore, Bedford, MA).

In all procedures involving diabetic animals, rats were fasted for a 24-hour period. Diabetes was then induced with a single 60 mg/kg intraperitoneal injection of streptozotocin (Sigma) in 10 mM citrate buffer, pH 4.5. Animals that served as nondiabetic controls received an equivalent amount of citrate buffer alone. Twenty-four hours later, rats with blood glucose levels greater than 250 mg/dl were deemed diabetic. Exactly 1 week later, just before experimentation, blood glucose levels were assayed again to confirm diabetic status.

Measurement of Plasma Evans Blue Concentration of Diabetic Animals and Nondiabetic Controls

One-week diabetic rats and age-matched nondiabetic controls were anesthetized with ketamine (Ketalar, 80 mg/kg; Parke-Davis, Morris Plains, NJ) and xylazine (Rompun, 4 mg/kg; Harver-Lockhart, Morris Plains, NJ). Additional anesthesia was provided throughout the procedures as needed. The right jugular vein and right iliac artery were cannulated with 0.28- and 0.58-mm internal diameter polyethylene tubing (Becton Dickinson, Sparks, MD), respectively, and filled with heparinized saline (±000 units heparin/ml saline). Evans blue was injected through the jugular vein over 10 seconds at a dosage of 45 mg/kg. Immediately after Evans blue infusion, the rats turned visually blue, confirming their uptake and distribution of the dye. Two minutes after the injection of Evans blue, 0.2 ml blood was drawn from the iliac artery to obtain the initial Evans blue plasma concentration. Subsequently, at 15-minute intervals, 0.1 ml blood was drawn from the iliac artery up to 2 hours after injection to obtain the time-averaged Evans blue plasma concentration. At exactly 2 hours after infusion, 0.2 ml blood was drawn from the left ventricle to obtain the final Evans blue plasma concentration. These blood samples were centrifuged at 12,000 rpm for 15 minutes and diluted to 1/10,000th their initial concentration in formamide. The absorbance was then measured with a spectrophotometer (model DU-640; Beckman, Fullerton, CA) at 620 nm, the absorption maximum for Evans blue dye in formamide. The concentration of dye in the plasma was calculated from a standard curve of Evans blue in formamide. Results are expressed in micrograms of Evans blue per microliter of plasma.

Measurement of VEGF-Induced, Blood–Retinal Barrier Breakdown Using Evans Blue

After induction of generalized anesthesia as outlined above, pupils were dilated with 0.5% tropicamide (Bausch & Lomb Pharmaceuticals, Tampa, FL). The vitreous of one eye was injected with 50 ng recombinant murine VEGF165 (R&D Systems Inc., Minneapolis, MN) in 5 μl PBS buffer using a 10-μl, 27-gauge Hamilton syringe (1701RN-80030; Hamilton Company, Reno, NV). The contralateral eye, which received an equivalent amount of citrate buffer alone, served as a control pair. Approximately 24 hours later, Evans blue was injected at a dosage of 45 mg/kg and blood samples were taken as described above. After the dye had circulated for 120 minutes, the chest cavity was opened, and rats were perfused via the left ventricle at 37°C with citrate buffer (0.05 M, pH 3.5). The perfusion lasted 2 minutes at a physiological pressure of 120 mm Hg. Immediately after perfusion, both eyes were enucleated and bisectioned at the equator. The retinas were then carefully dissected away under an operating microscope. After measurement of the retinal wet weight, retinal barrier breakdown was calculated using the following equation, with results being expressed in μl plasma × g retinal wet wt⁻¹·h⁻¹.

Measurement of Diabetes-Induced, Blood–Retinal Barrier Breakdown Using Evans Blue

The diabetes-induced, blood–retinal barrier breakdown experiments were slightly modified to increase both the sensitivity and reproducibility of the technique (see Discussion). After induction of generalized anesthesia as outlined above, Evans blue was injected at a dosage of 45 mg/kg, and blood samples were taken as described above. After the dye had circulated for 120 minutes, the chest cavity was opened, and rats were perfused via the left ventricle at 37°C with citrate buffer (0.05 M, pH 3.5). The perfusion lasted 2 minutes at a physiological pressure of 120 mm Hg. Immediately after perfusion, both eyes were enucleated and bisected at the equator. The retinas were then carefully dissected away under an operating microscope. After measurement of the retinal wet weight, retinas were thoroughly dried in a Speed-Vac (~5 h). The Evans blue dye was extracted by incubating each retina in 120 μl formamide (Sigma) for 18 hours at 70°C. The extract was ultra-centrifuged (Beckman TLX) at a speed of 70,000 rpm for 45 minutes at a temperature of 4°C. Sixty microliters of the supernatant was used for triplicate spectrophotometric measurements with each measurement occurring over a 5-second interval and all sets of measurements preceded by known standards. A background-subtracted absorbance was determined by measuring each sample at both 620 nm, the absorbance maximum for Evans blue, and 740 nm, the absorbance minimum. The concentration of dye in the extracts was calculated from a standard curve of Evans blue in formamide. Blood–retinal barrier breakdown was calculated using the same equation listed above, substituting dry weight for wet weight, with results being expressed in μl plasma × g retinal dry wt⁻¹·h⁻¹.

Statistics

Blood Evans blue concentration and VEGF- or diabetes-induced, blood–retinal barrier breakdown were analyzed with paired and unpaired two-tailed Student’s t-tests, respectively. All multiple comparisons used an analysis of variance (ANOVA). Differences were considered statistically significant if P < 0.05. All numerical results are expressed as means ± SE.

RESULTS

Demonstration of a Reliable Evans Blue Standard Curve at Concentrations Measured

Figure 1 demonstrates that even at levels approximately 10-fold lower than those measured in other tissues, Evans blue standard curves in formamide maintain a reliable linear relationship between background-subtracted absorbance (620–740 nm) and concentration from 25 to 1000 ng/ml (r = 0.999, Abs = 4 × 10⁻⁶ ± 0.0015). Standards run on different days (circles versus triangles) were virtually identical (r = 0.998).

Comparison of Evans Blue Plasma Concentration of Diabetic Animals and Nondiabetic Controls

Figure 2 shows the initial, time-averaged, and final Evans blue plasma concentrations (mean ± SE, μg Evans blue/μl plasma).
of diabetic animals and nondiabetic controls. The initial, time-
averaged, and final Evans blue concentrations in diabetic and
normal animals, respectively, were as follows: initial, 1.38 ±
0.10 and 1.38 ± 0.09 μg Evans blue/μl plasma (n = 6); time-averaged, 1.09 ± 0.09 and 1.05 ± 0.09 μg Evans blue/μl plasma (n = 6); and final, 0.89 ± 0.07 and 1.00 ± 0.07 μg
Evans blue/μl plasma (n = 6). The results show that the Evans
blue plasma clearance of 1-week diabetic animals and nondia-
betic controls does not differ significantly.

Quantitation of VEGF-Induced, Blood–Retinal Barrier Breakdown Using Evans Blue:
Comparison of Normal Saline, Citrate, or Paraformaldehyde Perfusion Solutions

Figure 3 shows the retinal Evans blue leakage (mean ± SE, μl plasma × g retinal wet wt⁻¹·h⁻¹) 24 hours after intravitreous injections of 50 ng VEGF (solid line) or saline (open circle). Data are means ± SE, with n representing the number of rats in each group. *Values significantly different from the corresponding control group.

Comparison of Dry and Wet Retina Weights

Figure 4 shows the degree of inter-retina variability (% difference in weight) between left and right retinal tissue weights when both dry weight (n = 74 retina pairs) and wet weight (n = 93 retina pairs) were compared for the diabetes studies. The experimental conditions were such that each eye received an identical treatment (i.e., streptozotocin-induced diabetes). Intra-animal, inter-retinal dry weight variability was found to be only 11.2% ± 1.0%, whereas inter-retinal wet weight variability was found to be 20.5% ± 1.6% (P < 0.05). Thus, dry retina weights demonstrated less variability than wet retina weights.
quantitation of diabetes-induced, blood–retinal barrier breakdown using evans blue

Figure 5 shows the retinal blood vessel leakage (μl plasma × g retinal dry wt⁻¹ · h⁻¹) of 1-week diabetic rats that received a single 60 mg/kg intraperitoneal injection of streptozotocin 1 week prior. Data for nondiabetic control rats, which received equivalent volume of citrate buffer only, is also displayed. The mean ± SE of retinal Evans blue leakage was 5.36 ± 0.80 (n = 10 retinas) and 9.28 ± 0.90 (n = 11 retinas) μl plasma × g retinal dry wt⁻¹ · h⁻¹ for nondiabetic and diabetic rats, respectively (P < 0.05). Thus, the retinal Evans blue leakage of 1-week diabetic rats was approximately 1.7-fold higher than that of nondiabetic control rats.

Discussion

Until now, Evans blue has not been sensitive and reliable enough to quantitate blood-retinal barrier breakdown, such as occurs in diabetic retinopathy and multiple other eye diseases. In the present study, VEGF was used to rapidly induce extensive breakdown of the blood-retinal barrier, and the ability of a modified Evans blue dye technique to quantify this breakdown was assessed. The results showed that the retinal Evans blue leakage in eyes injected with VEGF was significantly higher than that of paired control eyes, regardless of the perfusion solution used. With citrate as the perfusion solution, a 4.0-fold difference was noted. These results with VEGF established that the Evans blue technique can quantify relatively high levels of blood-retinal barrier breakdown. They did not demonstrate whether the technique is sensitive enough to detect the less robust blood-retinal barrier breakdown that characterizes early diabetic retinopathy. For this purpose, 1-week diabetic animals and age-matched nondiabetic controls were used. The results showed that the leakage of retinal Evans blue in diabetic animals is 1.7-fold higher than that in normal controls. Although it might be expected that the increased retinal permeability state of diabetic animals would result in greater clearance of Evans blue from the plasma, Figure 2 demonstrates that this is not the case. This is because the total amount of Evans blue extravasated in the retina is extremely small in comparison to the total amount present in the bloodstream.

The experiments with VEGF and 1-week diabetic animals are in close accordance with published isotope-dilution studies. In studies using the isotope-dilution method, 125I-bovine serum albumin (BSA), as opposed to Evans blue, is introduced through the jugular vein and allowed to circulate in the bloodstream as an intravascular tracer. As with the Evans blue technique, whenever plasma extravasates from blood vessels, the 125I-BSA leaks into the surrounding tissues. Throughout the isotope-dilution experiments, blood is withdrawn from the iliac artery to determine the time-averaged 125I-BSA plasma activity as opposed to time-averaged Evans blue concentration. After a circulation time of approximately 8 minutes, a second tracer, 131I-BSA, is introduced through the jugular vein to correct the 125I-BSA retinal activity for 125I-BSA that remains in the retinal vasculature. A similar correction is not needed with the Evans blue technique because remaining intravascular tracer is cleared from the bloodstream during perfusion. However, unlike the isotope-dilution technique, extravasated Evans blue must be extracted with formamide to allow its quantitation. Two minutes after introduction of 131I-BSA, a final blood sample was obtained, and the retinas were quickly excised and weighed. A γ spectrometer was then used to measure the 125I and 131I radioactivity in the retinal tissues and in both the time-averaged and final blood samples. By comparison, the Evans blue technique uses a routine spectrophotometer to measure Evans blue absorbance. Final results using the isotope-dilution method express blood–retinal barrier breakdown in μg plasma × g retinal dry (or wet) wt⁻¹ · h⁻¹. By comparison, with the Evans blue technique final results are expressed in μl plasma × g retinal dry (or wet) wt⁻¹ · h⁻¹.

The results using the Evans blue technique compare quite favorably with similar studies using the isotope-dilution method. With citrate as the perfusion solution, the Evans blue technique quantified a 4.0-fold increase in retinal Evans blue leakage in eyes injected with VEGF compared with paired controls.
control eyes. This result is in close accordance with similar isotope-dilution experiments demonstrating a 3.2-fold increase in $^{125}$I leakage in eyes injected with VEGF. The 1-week diabetic experiments using the Evans blue technique likewise compare favorably with similar studies using the isotope-dilution method. The Evans blue technique quantified a 1.7-fold increase in retinal Evans blue leakage in 1-week diabetic animals compared with age-matched nondiabetic controls. This result is again in accordance with similar isotope-dilution experiments demonstrating a 2.9-fold increase in $^{125}$I leakage in 1-week diabetic animals.

In the experiments on 1-week diabetic animals, because each animal could no longer serve as its own control, several steps were modified to increase the overall sensitivity and reproducibility of the technique. First, the dye dosage was increased to 45 mg/kg, which based on previous studies in rats, does not exceed the binding capacity of plasma albumin (at least 200 mg/kg). The binding capacity is an important consideration because any changes in plasma albumin content due to the induction of diabetes might bias the results if the binding capacity were approached by the administered dye dosage. Second, on the basis of previous studies that demonstrated increased dye leakage as a function of time, the Evans blue circulation time was prolonged to 2 hours. Third, retinal blood vessel leakage was normalized against retinal dry weights. This was done because retinal wet weights from a given animal sometimes vary considerably. This variability could be due to small amounts of vitreous sticking to the retina on its removal from the eye. When dealing with such small absolute tissue masses, small changes in hydration can introduce significant variability. Because the vitreous is predominantly water, upon drying, all the water evaporates. Fourth, the volume of formamide was reduced to 120 μl to increase the sensitivity of the Evans blue absorbance readings. Fifth, each sample was read in triplicate at 620 nm, the absorbance maximum of Evans blue in formamide, and at 740 nm, an absorbance minimum. This was done because in the initial experiments, it was noticed that reading the same sample twice sometimes gave slightly different results. This was because even minimal misalignment of the spectrophotometer's cuvette changes the path length of light and thus the absorbance measurement obtained. By reading at two different wavelengths, however, and calculating the background-subtracted absorbance, the results became extremely consistent because positioning changes affect both absorbance measurements to the same degree, thus enabling the positioning artifact to be factored out. These modifications were critically important in adapting and optimizing the technique in the diabetic retina, where leakage is an order of magnitude lower than previously studied nonocular tissues. In the trachea, for example, leakage can reach up to 135 ng/mg, and in the conjunctiva, 111 ng/mg. However in our first study, Evans blue leakage in VEGF-treated retinas was less than 7 ng/mg wet wt (citrate diffusion), representing recovery of only 0.015% of the total injected Evans blue. This may be partially explained by the fact that the blood–retinal barrier has tight junctions, although they are breached in this context.

In conclusion, we have shown that Evans blue dye can serve as an effective alternative to the isotope-dilution method for sensitively quantitating blood–retinal barrier breakdown. It has numerous advantages over the isotope-dilution method. It is much safer to use as it does not involve highly radioactive isotopes, is considerably simpler to use because it has no parallel to the complex preparatory albumin–iodination process, and is much less expensive because a 50-g annual supply costs only $80. The method has broad applicability to numerous diseases that affect the blood–retinal barrier and should help speed the development of therapeutic drugs that reconstitute the barrier in disease. Future experiments should involve perfecting the technique in the murine model to enable experiments with genetically-altered mice. The possibility of correlating Evans blue spectrophotometric quantitation with its florescence histologically should also be investigated.

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**References**