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Photodynamic Retinal Vascular Thrombosis

Rate and Duration of Vascular Occlusion

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Dye-sensitized photochemical thrombosis is a new method of producing vascular occlusion in the eye for experimental purposes. The rate and duration of photodynamic occlusions of branch retinal vessels was measured in pigmented and albino rat eyes after intravenous injection of the photosensitizing dye, rose bengal. Selected vessels were exposed to focused, white light until vascular occlusion was observed biomicroscopically. A slit lamp was used for a light source in this procedure, allowing adjustment of spot size, shape, and orientation. Arterioles occluded more rapidly than venules, and the time required to produce vascular occlusion decreased when animals breathed pure oxygen administered by face mask. Rose bengal doses of 40 and 80 mg/kg were effective, 20 mg/kg was partially effective, and 1 and 10 mg/kg were ineffective in producing branch arteriole occlusion at a light intensity of 73.5 mW/cm^2. The total light energy required to produce occlusion increased from an average of 0.06 J using 80 mg/kg to 0.50 J using 20 mg/kg of rose bengal. Lower light intensities produced vessel occlusion less rapidly (46 mW/cm^2) or not at all (17.5 mW/cm^2). The rate of retinal arteriolar occlusion was not affected by ocular pigmentation. The duration of branch vessel occlusion depended on length of vessel treated and did not exceed 3 days in arterioles and 4 days in venules. Histologic sections showed discrete areas of retinal and choroidal vascular thrombosis confined to the area of direct light exposure. Choroidal vascular thrombosis and outer retinal damage predominated in eyes treated at low light intensity. Thrombosis usually extended into the deep choroidal vessels in albino but not pigmented eyes. These results provide a basis for the use of photodynamic thrombosis in modeling retinal branch vessel occlusion. Such a model may be useful in evaluating potential treatments of retinal ischemia. Invest Ophthalmol Vis Sci 32:2357-2365,1991

Retinal vascular occlusive disease is a common cause of visual impairment and blindness. Investigations into the prevention or treatment of ischemic retinal damage are furthered by various animal models of retinal vascular hypoperfusion. Recently, we[1,2] and others[3] described a new method of producing experimental thrombosis of retinal vessels. In this method, a photosensitizing agent is injected intravascularly and activated in the eye by visible light. Dye activation in the presence of molecular oxygen leads to the generation of singlet oxygen, which, in turn, acts locally to injure or destroy the vascular endothelium. The altered endothelium provides a surface for platelet aggregation and subsequent thrombosis. Because relatively low light intensities are required to initiate this process, thermal effects on surrounding tissues can be minimized. In this study, the photosensitizing dye rose bengal was chosen because of its high photochemical efficiency and low systemic toxicity.

The photobiologic properties of rose bengal have been established in various ocular and nonocular tissues,[4-10] but its use in producing retinal vascular occlusion has not been evaluated quantitatively. Therefore, we undertook this investigation to describe the characteristics of photothrombotic occlusion of branch retinal vessels in the rat, with attention to factors that could influence the rate or duration of occlusion. Light and electron microscopic studies were done to support the clinical findings. The results provide a framework for future applications of this technique.

Materials and Methods

Male Sprague-Dawley albino and ACI pigmented rats, weighing 200–250 g, were obtained from Harlan Sprague-Dawley (Indianapolis, IN). These studies were done in accordance with the ARVO Resolution on the Use of Animals in Research. Institutional

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guidelines regarding animal experimentation were followed. In preparation for treatment and follow-up examinations, the rats were anesthetized by intramuscular injection of ketamine HCl (80 mg/kg) and xylazine HCl (8 mg/kg). Their pupils were dilated using 1% cyclopentolate drops.

Treatment and observation was done using a Topcon photo slit lamp (model SL-5D; Paramus, NJ). The light intensity of the slit lamp was measured using a YSI-Kettering model 65A radiometer (Yellow Springs Instrument, Yellow Springs, OH) on several occasions throughout the study. This was accomplished by orienting the slit beam perpendicular to and focusing a 5-mm diameter circular spot on the sensor element. The heat absorption filter in the slit lamp was used at all times. Light intensities averaged 73.5, 46, and 17.5 mW/cm^2, for the high, medium, and low settings, respectively, with less than 3% variation on repeated measurements throughout the study.

Rose bengal (tetrachloro-tetraiodo-fluorescein sodium, certified purity, 90%; Sigma, St. Louis, MO) was dissolved in normal saline (0.5, 5, 10, 20, or 40 mg/ml), sterilized by passage through a 0.22-μm filter, and injected in doses of 1, 10, 20, 40, or 80 mg/kg before light treatment. Rose bengal injections were done through the dorsal penile vein, and light exposures were begun 1 min later to allow time to position the animal for treatment.

In the treatment protocol, the rats were placed on a stage mounted to the slit lamp with one eye lightly touching a fixed glass cover slip. Methylcellulose solution (2%) was used to couple the cover slip optically to the cornea. Through the biomicroscope, fundus details could then be visualized and photographed. Depending on the experiment, the light beam was set to deliver a circular spot of illumination (1-mm diameter) or narrowed to a slit beam just wide enough to cover the target vessel completely (Fig. 1). In the latter case, varying lengths of vessel could be treated by choosing the 1-, 2-, or 3-mm settings. Treatment of relatively large vessel lengths was facilitated by the radial, spoke-like arrangement of the retinal vasculature. Vessels were treated in such a manner that one edge of the light beam was as close to the optic disc as possible, taking care to avoid exposing adjacent vessels. In portions of the study, 100% oxygen was delivered by a face mask (devised from a 60-ml syringe barrel), before and during treatment. The flow rate was set at 10 l/min to maximize the concentration of oxygen breathed.

The end point of treatment was branch vessel occlusion or, in the absence of occlusion, observation for 20 min during which the vessel was exposed to light continuously. Vessel occlusion was recognized by the biomicroscopic appearance of stagnant red cells in the vessel at the time of their first appearance in or around the area of thrombosis. Occlusion followed the formation of intravascular plaque (throm-

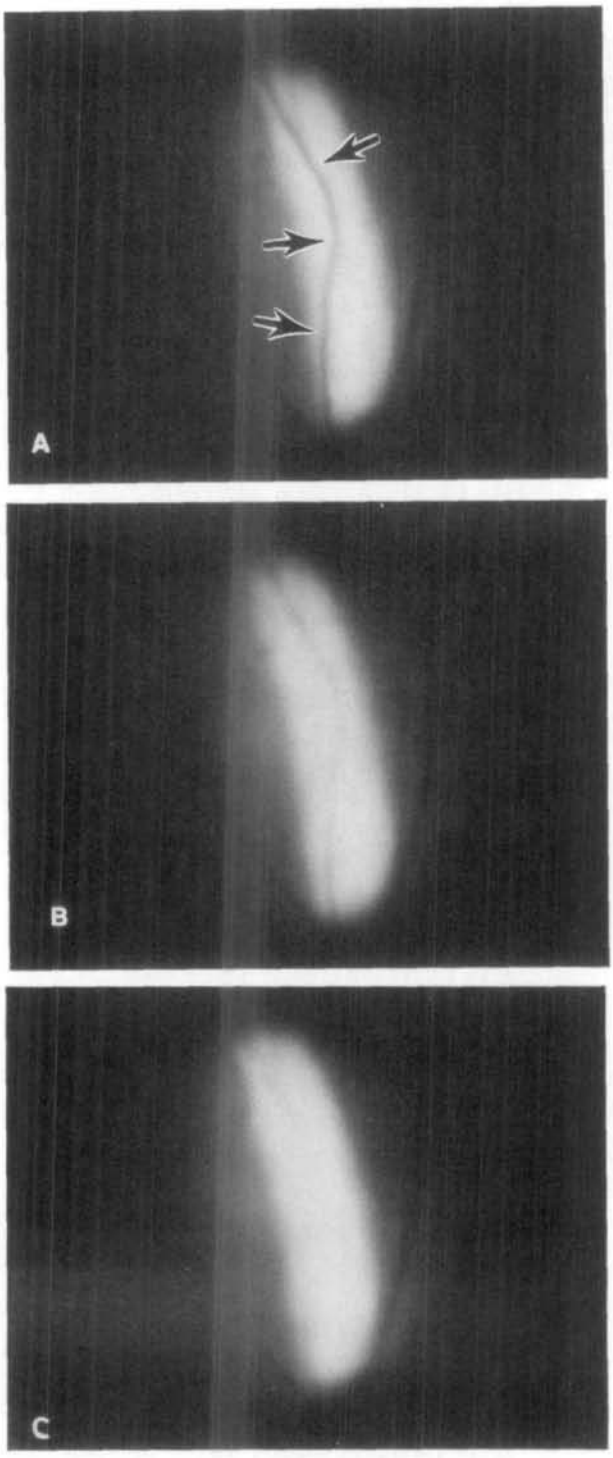

Fig. 1. Photodynamic thrombosis of a retinal venule in an albino eye. The slit beam is 3 mm in length. Initial formation (A) of intravascular plaque-like thrombi (presumably composed of platelets) can be seen by irregularities in the blood column (arrows) followed by (B) and (C) progressive obliteration of the vessel lumen.

bus), which was seen as white debris in the vessel lumen.

Special attention was given to maintaining a constant interval (1 min) between injection and onset of light exposure because rose bengal clearance from the blood has been shown to be rapid in rats and other animals.[11] To evaluate the effect of rose bengal clearance on photothrombosis, a preliminary study was done in which the time between injection of rose bengal and onset of light exposure was varied (1, 10, 20, 40, or 60 min). Branch retinal arterioles in albino rats were occluded reliably up to 20 min after injection, using a light intensity of 73.5 mW/cm^2 (1-mm circular spot) and a rose bengal dose of 40 mg/kg. The speed of occlusion, however, decreased with time. Vascular occlusion was not produced consistently after 40 or 60 min.

The duration of branch vessel occlusion was monitored by daily observation until reperfusion was noted clinically. Reperfused vessels were characterized by an uninterrupted blood column and the absence of stagnant red blood cells. Blood flow was confirmed by transiently exerting pressure on the eye, reducing the flow rate until freely moving red cells were seen. When reperfusion was noted on posttreatment day 1, the vessel was assigned 0 days of occlusion. When occluded on day 1, but not on day 2, the vessel was assigned 1 day of occlusion, and so forth.

Selected eyes were examined histologically (1 or 3 days posttreatment) after perfusion–fixation of the animals with a 1:1 mixture of 4% paraformaldehyde and 5% glutaraldehyde. Perfusion–fixation was done through the left ventricle after cross-clamping the descending aorta, incising the inferior vena cava, and

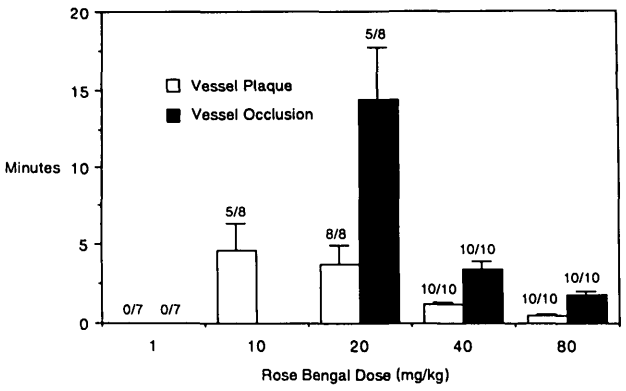

Fig. 2. Effect of dose of rose bengal on the time required to produce visible thrombus (plaque) or occlusion of retinal arterioles in albino rats (mean, SE). Increasing dose accelerated occlusion (r^2 = .54 and $P < 0.001$). The number of vessels responding to treatment (numerator) and the total number of vessels treated (denominator) are shown above each column. All light exposures were performed using a 1-mm circular spot size at a light intensity of 73.5 mW/cm^2.

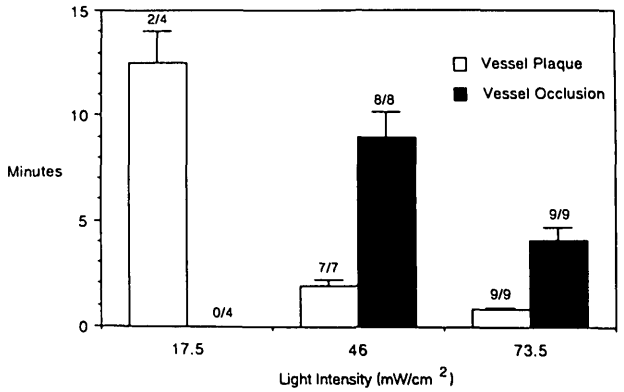

Fig. 3. Effect of light intensity on the time required to produce a visible thrombus (plaque) or occlusion of retinal arterioles in albino rats (mean, SE). Increasing light intensity from 46 to 73.5 mW/cm^2 accelerated vessel occlusion (P = 0.002, students t-test). The number of vessels responding to treatment (numerator) and the total number of vessels treated (denominator) are shown above each column. All light exposures were performed using a 1-mm circular spot size and a rose bengal dose of 40 mg/kg.

infusing cold saline. Enucleated eyes were placed in fixative for 1 hr. Then the eye was dissected and allowed to fix overnight. Tissue specimens were then processed in 2% buffered osmium tetroxide for 90 min, washed three times in phosphate buffer, dehydrated in increasing concentrations of ethyl alcohol, and embedded in low-viscosity epoxy resin. Thick sections for light microscopy were stained with methylene blue and basic fuchsin. Thin sections for electron microscopy were stained with uranyl acetate–lead citrate.

Results

Photodynamic injury resulted in the gradual accumulation of white debris (plaque-like thrombi) in the vessel followed by vascular occlusion (Fig. 1). Occlusion of smaller vessel branches and the appearance of an underlying area of choroidal "whitening" (best seen in albino rats) generally preceded occlusion of the targeted vessel. Arterioles responded to treatment with a brief period of vasoconstriction followed by vasodilation, both of which preceded vessel thrombosis. Venules showed engorgement proximal to the occlusion site. Venular occlusion was associated with the gradual appearance of blot retinal hemorrhages peripherally.

Dose- and Light-Response Studies

Arteriolar occlusion in albino rats was used as the end point for the determination of dose- and light-response relationships. In these studies, the slit lamp was set to deliver a 1-mm circular spot. The time required to produce occlusion decreased with increasing dose of rose bengal and light intensity (Figs. 2, 3).

Doses of 1 and 10 mg/kg were ineffective, 20 mg/kg was partially effective, and 40 and 80 mg/kg were highly effective in producing target vessel occlusion at a light intensity of 73.5 mW/cm^2. At a dose of 1 mg/kg, no clinical changes were seen. Small vascular side branches became occluded, and the underlying choroid became blanched at doses as low as 10 mg/kg. At that dose, small plaques also developed in the target vessel, but that did not progress to vascular occlusion. The total dose of light energy required to produce target vessel occlusion averaged 0.50, 0.12, and 0.06 J, using rose bengal doses of 20, 40, and 80 mg/kg, respectively.

In the light intensity–response study, the dose of rose bengal was kept constant (40 mg/kg). The highest light intensity, 73.5 mW/cm^2, produced arteriolar occlusion in less than one-half the time required at 46 mW/cm^2 (Fig. 3). The lowest light intensity, 17.5 mW/cm^2, did not produce target vessel occlusion but caused choroidal whitening, small retinal vessel occlusion, and abortive plaque formation.

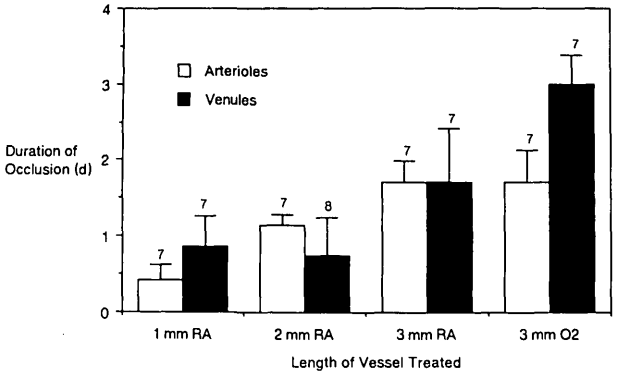

Fig. 5. Relationship between vessel length treated (according to slit-lamp setting) and duration of vessel occlusion while breathing room air (RA) or oxygen (O_2). The number of observations is shown above each column. Increasing the size of the vessel lesion significantly prolonged the occlusion of arterioles ($r^2 = 0.49$; $P < 0.001$) but not venules ($r^2 = 0.06$; $P = 0.29$). Oxygen breathing did not significantly alter the duration of vascular occlusion. Combined oxygen breathing and large venular lesions (3 mm setting) produced significantly longer occlusions than small (1 mm) lesions ($P = 0.002$; student t-test).

Effect of Oxygen Breathing

Branch retinal vessels in albino rats were treated with a 1-mm circular spot size at an intensity of 73.5 mW/cm^2 after a rose bengal dose of 40 mg/kg. The time required to produce occlusion in animals breathing room air was compared with that of animals breathing pure oxygen through a face mask. Photodynamic occlusions were significantly accelerated in both arterioles and venules by increasing the concentration of inspired oxygen (Fig. 4). To confirm that the rats were being oxygenated by the face mask apparatus, arterial blood gases were analyzed on blood drawn from the left ventricle of selected animals. The Po_2 increased from 39 ± 9.3 mm Hg room air to 172 ± 54 mm Hg (n = 5; $P = 0.04$) with oxygen breathing.

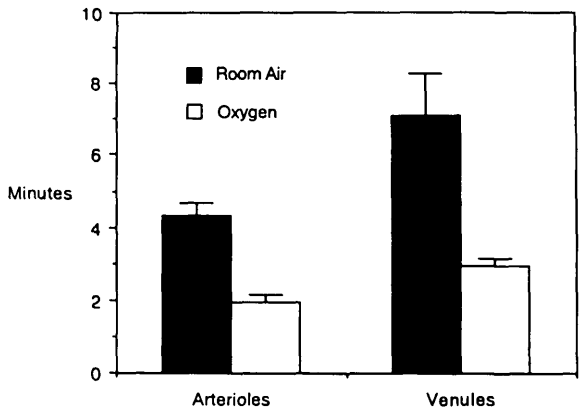

Fig. 4. Comparison of time required to produce branch vessel occlusion in albino rats breathing oxygen by face mask versus room air (mean, SE, n = 8 for all values). Oxygen breathing significantly reduced vascular occlusion times ($P < 0.001$ and = 0.002 for arterioles and venules, respectively; student t-test). Arterioles occluded more rapidly than venules in either condition ($P = 0.036$ and = 0.005 for room air and oxygen breathing, respectively).

Effect of Ocular Pigmentation

The speed of photodynamic retinal arteriolar occlusion was compared in eight pigmented (ACI) and eight albino rats using a 1-mm circular spot size, an intensity of 73.5 mW/cm^2, and a rose bengal dose of 40 mg/kg. In both groups, the mean time of arteriolar occlusion was the same (4.4 ± 0.4 min; mean ± standard error).

Duration of Occlusion

Preliminary experiments suggested that photodynamic occlusions of small lengths of vessel (1 mm or less) were relatively short-lived. Therefore, this study evaluated the effect of treating longer lengths of vessel (1-, 2-, or 3-mm slit lamp settings) on the duration of arteriolar and venular occlusion. Because oxygen breathing accelerated vascular occlusion in these studies, we also examined its effect on the duration of occlusion using the 3-mm setting. All treatments were conducted in albino rats using a light intensity of 73.5 mW/cm^2 and a rose bengal dose of 40 mg/kg.

Increasing the length of vessel treated significantly prolonged the occlusion of arterioles but not venules (Fig. 5). Oxygen treatment did not increase the duration of occlusion after lesions of comparable length. The duration of venular occlusion, however, was sig-

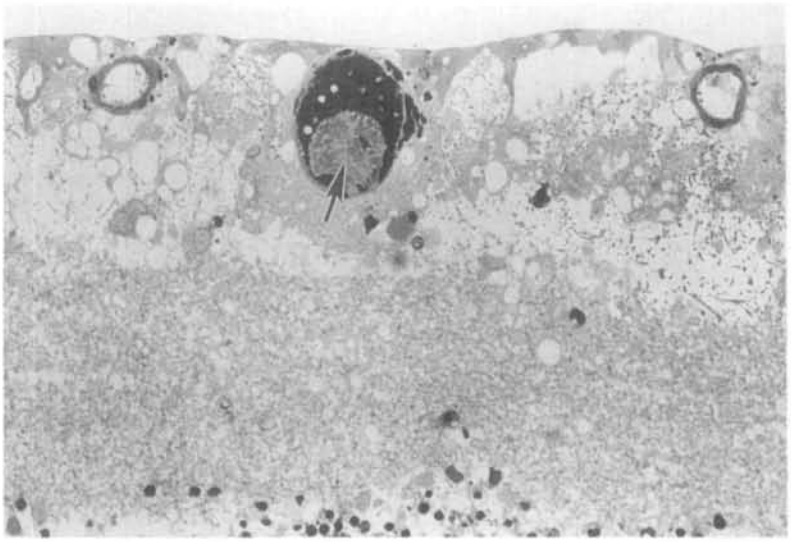

Fig. 6. Light micrograph of retina 1 day after photodynamic vascular occlusion using a light intensity of 73.5 mW/cm^2, a light dose of 0.14 Joules and a rose bengal dose of 40 mg/kg. The target retinal venule is partly occluded with a plaque (arrow) which bulges into the vessel lumen. Stagnant red blood cells occupy the remainder of the lumen. Adjacent areas of inner retina show evidence of degeneration (methylene blue, basic fuchsin; magnification ×100).

nificantly greater in large lesions (3-mm setting) produced during supplemental oxygen breathing compared with small lesions (1-mm setting) produced while breathing room air. Permanent vascular occlusion was not observed. The longest occlusions obtained under any condition were 3 days in arterioles and 4 days in venules.

Histologic Findings

Histologic sections were used to confirm the presence and extent of photodynamic lesions. Retinal and choroidal damage was confined to the area of direct light exposure (Figs. 6–8). Adjacent areas and sections obtained away from the lesion (opposite the optic disc) showed no photodynamic damage. Retinal vessels were occluded primarily with platelets and stagnant red blood cells, although white blood cells were occasionally observed (Fig. 7). Surrounding areas of retina showed degenerative changes consistent with ischemia. In most lesions, however, the outer retinal damage exceeded that of the inner retina. This was particularly noticeable in lesions obtained from treatments that did not produce clinical retinal branch vessel occlusion (Fig. 8). Such lesions showed primarily choroidal vascular occlusion with marked disruption of the outer retinal layers.

In the choroid, the effect of photothrombosis was influenced by ocular pigmentation (Fig. 9). In pigmented rats, deep choroidal vessels did not develop thrombosis to the extent seen in albinos. In albino eyes, thrombosis was noted in the deep choroid and sometimes in vessels traversing the sclera. Subretinal exudates composed of cellular debris, retinal pigment epithelial cells, macrophages, and a variable amount of proteinaceous fluid were also more extensive in albino eyes.

Control eyes in albino rats were exposed to the highest light intensity (73.5 mW/cm^2) for 20 min without the administration of rose bengal; these showed no histologic evidence of photochemically or thermally induced retinal damage.

Discussion

Photochemical retinal vascular thrombosis was produced in the rat to examine the rate and duration of occlusion under various conditions. The findings demonstrated a dose-, light-, and oxygen-dependent effect on the rate of thrombosis. These effects are well-established characteristics of photosensitization reactions that have been studied in other models,[9,10,12–15] but they have not been examined quantitatively with respect to the production of retinal vascular thrombosis. A similar method of producing photothrombotic ischemia of the rat retina was reported using rose bengal.[3] These authors consistently produced ischemic damage in albino rats using a rose bengal dose of 40 mg/kg but reported that a higher dose (80 mg/kg) was required to produce comparable effects in pigmented rats. One reason for their observation may have been that the projection light source used in their study was "focused on the eye" rather than through the pupil. Therefore, differences in light attenuation due to iris pigmentation may have resulted. In our study, a rose bengal dose of 40 mg/kg appeared equally effective in producing vascular occlusion in pigmented and albino rats when light intensity and other treatment parameters were controlled.

Retinal venular occlusion consistently proceeded at a slower rate than arteriolar occlusion. Although the reason is uncertain, we suspect that blood oxygen satu-

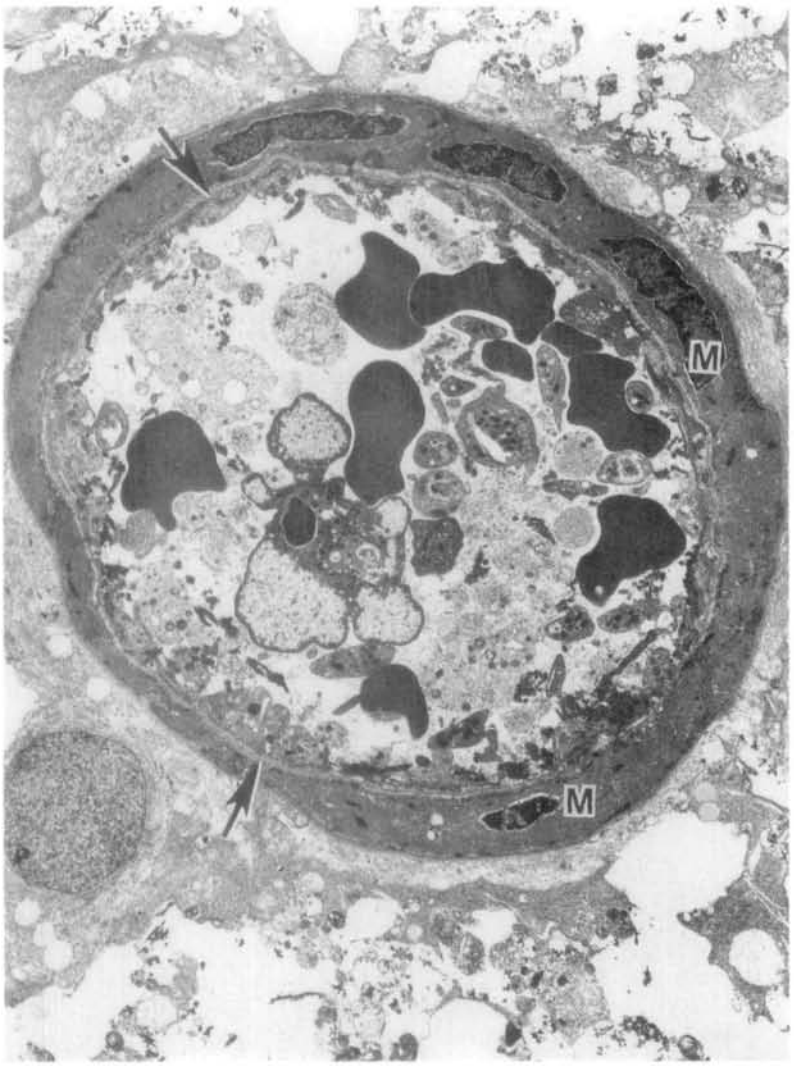

Fig. 7. Electron micrograph shows photodynamically occluded retinal vessel 1 day after treatment. Platelets line the inner vascular wall that is denuded of endothelial cells (arrows). In the lumen, platelets, red blood cells, and a single white blood cell are seen. Vascular smooth muscle cells (M) appear undamaged; (magnification ×2000).

ration may account in part for this difference. Human and animal studies have shown that the oxygen content of retinal venous blood is on the order of 38% lower than arterial blood,[16] a difference that could affect the rate of photochemical reaction. When blood oxygen concentration was increased by oxygen breathing, thrombosis was accelerated, particularly in venules.

By contrast, the choroidal arteriovenous oxygen difference is suspected to be much smaller than in the retina[16] as a result of low oxygen extraction and high blood flow in the choroid. In the cat, oxygen tension (Po_2) averages between 25 and 29 mm Hg in the retina but increases to a mean of 72 mm Hg at the level of the choriocapillaris.[17] Photochemical thrombosis, therefore, may be facilitated in choroidal tissues by a high oxygen concentration, which could explain the rapid onset of choroidal blanching observed in albino eyes during treatment.

Choroidal vascular thrombosis was noted in all histologic sections from treated areas, regardless of the severity of retinal vascular changes. Choroidal damage was, however, least pronounced in the eyes of pigmented rats. In these eyes, thrombosis did not regularly extend into the deep choroid as found in albino eyes. One possible explanation is that light absorption by melanin in the retinal pigment epithelium and choroid produced a substantial protective effect in pigmented eyes.

In addition to its high Po_2, the choroid possesses a highly permeable capillary network that may allow rose bengal to leak into and become trapped in the extravascular space. This tissue penetration could spread the photosensitizing effect over a greater length of time and cause direct photodynamic injury to perivascular tissues such as the retinal pigment epithelium. Rose bengal is over 99% protein bound in serum,[18] but choroidal vascular leakage could still occur if bound to albumin or smaller proteins.

Unlike the choroid, retinal blood vessels are rela-

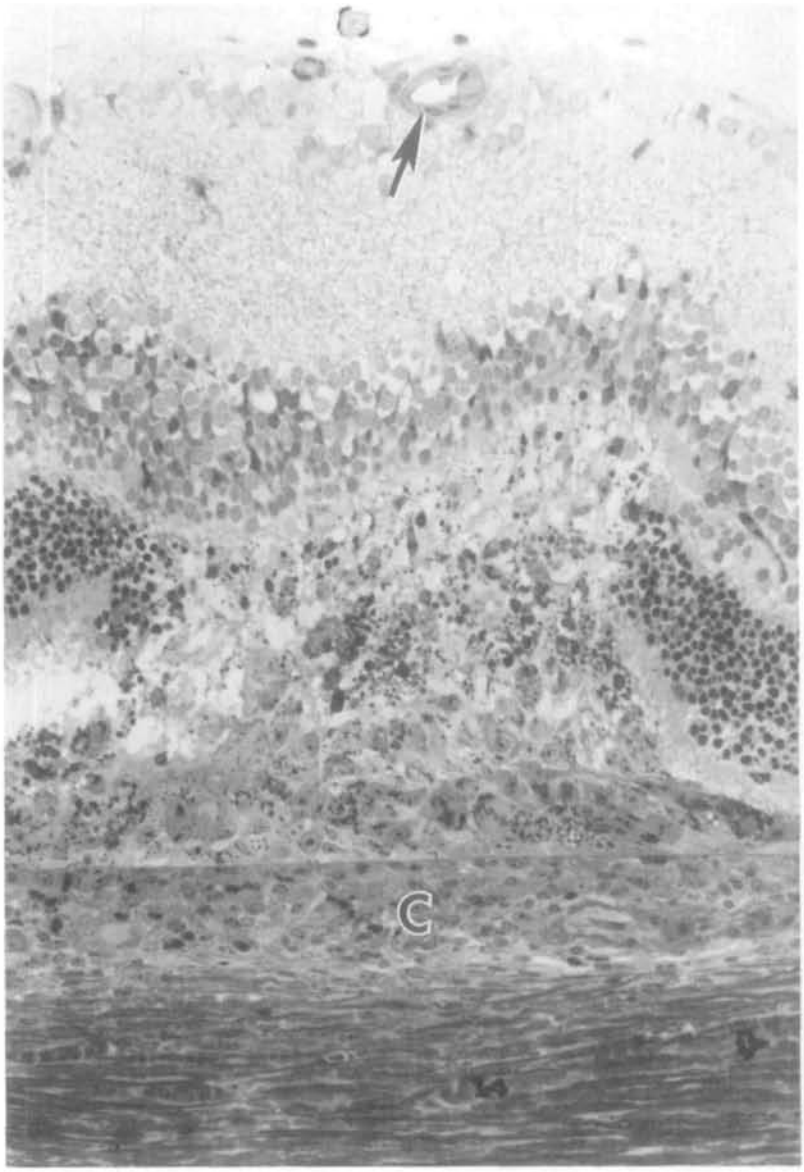

Fig. 8. Light micrograph of retina from albino eye treated 3 days previously at low light intensity (17.5 mW/cm^2), using a rose bengal dose of 40 mg/kg. Photodynamic thrombosis is seen in the choroid (C), but not in the target retinal vessel (arrow). In the choroid, no patent vessel can be found. The outer retinal damage is marked whereas the inner retina appears to be well preserved (methylene blue, basic fuchsin; magnification ×120).

tively impermeable to dyes such as rose bengal, particularly in their protein-bound state. Photodynamic injury, therefore, occurs initially at the luminal surface of vascular endothelial cells in the retina. During the course of treatment, increasing endothelial cell injury could allow leakage of rose bengal into the extravascular space. Dye might also gain access to the retina from the choroid through damaged retinal pigment epithelium. In a previous study using rose bengal, we found evidence of acute photodynamic damage in the retinal pigment epithelium of the cat eye.[19] Others also demonstrated photocytolysis of cultured retinal pigment epithelium cells in the presence of micromolar quantities of rose bengal.[12] Therefore, we cannot exclude the possibility of a direct photodynamic action on retinal cells outside the blood vessels.

Photodynamic retinal vascular occlusions were found to be relatively short term in this model (lasting up to 3–4 days). The duration of occlusion increased somewhat with the length of vessel treated (size of the lesion) but was not affected significantly by oxygen breathing. This would suggest that the duration of vascular occlusion was independent of the rate at which occlusion was produced. After thrombosis, vessel injury probably was limited by localized hypoxia, which removed the substrate for production of singlet oxygen.

Other studies used the argon laser or a spectrally filtered xenon-arc light source to produce photodynamic vascular occlusion in the eye.[1,2,7,19] We selected an immediately available light source: an ophthalmic slit lamp. The slit lamp presented minimal risk of

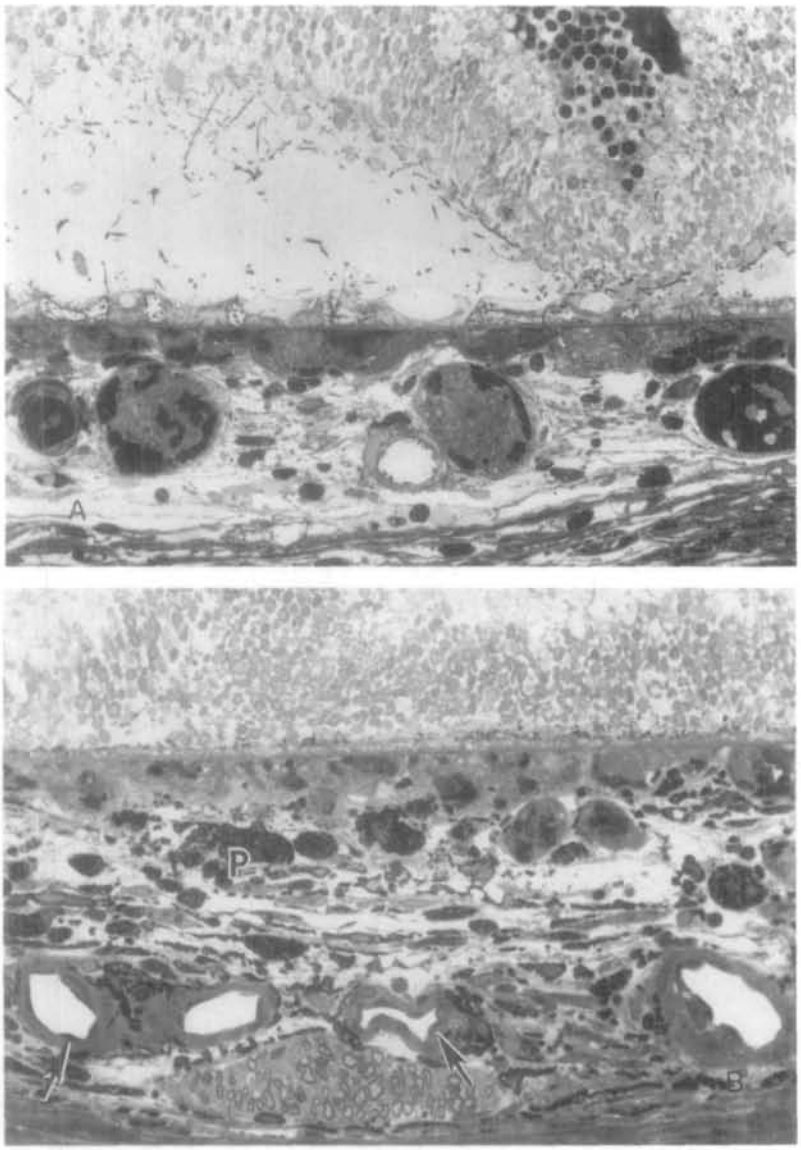

Fig. 9. Choroidal damage in albino rat 1 day after photodynamic thrombosis (**A**) shows endothelial damage and thrombosis of large and small choroidal vessels. Choroidal damage produced in pigmented rat (**B**) using identical treatment parameters (duration of treatment 4 min, dose of rose bengal 40 mg/kg, light intensity 73.5 mW/cm^2) shows preservation of the endothelial cells in deep choroidal vessels (arrows). A densely pigmented layer (P) separates damaged choriocapillaris from the undamaged vessels; (methylene blue, basic fuchsin; magnification ×400).

thermal injury to the retina in its operating range and permitted adjustment of spot size, shape, and orientation. The precise delivery of relatively low-intensity illumination allowed the production of well-defined lesions without incurring widespread retinal or choroidal damage.

Rose bengal has two broad absorption peaks in the green and yellow range of the spectrum,[13,14] both within the light output of the slit lamp. A potential disadvantage, however, in the use of white light sources is a fluctuation in intensity or color balance during the life span of the bulb. In our study, this problem was avoided by doing comparison studies on the same day and alternately treating animals from each group. Undesirable thermal effects were avoided by using the heat absorption filter included with the instrument.

In conclusion, we quantitatively examined many of the factors that influence the rate and duration of photodynamic retinal vascular occlusion. Photosensitizing agents are of increasing interest in vision research, both for developing models of retinal ischemia and evaluating the potential of the technique itself in treating vascular diseases of the eye. Our findings provide a basis for the development of these studies.

Key words: photodynamic thrombosis, rose bengal, rat, retinal vessels, dose response

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