Experimental Obstruction to Aqueous Outflow by Pigment Particles in Living Monkeys

David L. Epstein, Thomas F. Freddo,* P. John Anderson, Mary M. Patterson, and Susan Bossert-Chu

Pigment particles (1 × 10^6/μl) isolated from the iris and ciliary body of enucleated cynomolgus monkey eyes were infused into the anterior chamber of seven living cynomolgus monkeys and aqueous humor outflow facility determined by the two-step constant-pressure perfusion technique. Outflow facility acutely decreased 64% in the experimental pigment perfused eyes compared to a 76% increase in the sham-manipulated fellow eyes (P < 0.001). However, when next measured 1 wk later, facility in the pigment perfused eyes had returned to baseline levels. Repetitive pigment perfusions similarly failed to produce any long-term abnormality in outflow facility. Gonioscopically, a well-defined band of pigment was observed in the trabecular meshwork, which decreased in density with time. Scanning and transmission electron microscopy documented pigment particle phagocytosis by trabecular endothelial cells and macrophages. Forty-two and 105 days after pigment infusion the trabecular meshwork was normal morphologically, and, despite an observed decrease in trabecular pigmentation, morphometry failed to reveal a decrease in trabecular meshwork cellularity in experimental compared to control eyes. These results suggest that factors other than, or in addition to, pigment particle accumulation in the trabecular meshwork must be involved in the mechanism of human pigmentary glaucoma. Invest Ophthalmol Vis Sci 27:387-395, 1986

In human pigmentary glaucoma pigment lost from the peripheral posterior iris surface accumulates within the trabecular meshwork.1-8 There has been considerable discussion as to the mechanism for the associated glaucoma.1,3,9-20 Some have proposed that the glaucoma is unrelated to the pigment accumulation, representing instead a developmental angle abnormality14,15 or even primary open angle glaucoma.10 Others have argued that the pigment deposition itself in the trabecular meshwork results in a form of secondary open angle glaucoma.1,3,9,11,17,18

It is known that in excised eyes, perfusing pigment particles into the anterior chamber results in an acute obstruction of aqueous humor outflow.21 Similarly, it has been observed clinically that pigment particle liberation into the anterior chamber, either spontaneous, exercise-induced, or induced pharmacologically, can be associated with an acute elevation of intraocular pressure.19,22,23 However, what has been lacking is a chronic model of “pigmentary dispersion” in living animals in order to assess the longer term biological consequences of such pigment particle accumulation. We report here such a model of pigment dispersion in the living cynomolgus monkey.

Materials and Methods

Adult cynomolgus monkeys, weighing 2 kg to 3.5 kg, were used. The investigation conformed to the ARVO Resolution on the Use of Animals in Research. Preperfusion goniophotographs were taken using a pediatric Koepp lens (Ocular Instruments, Inc.; Redmond, WA) and a Kowa (Kowa Company, Ltd.; Tokyo, Japan) fundus camera. At the same time intracocular pressures were determined with a Digilab pneumotonometer (Digilab, Inc.; Cambridge, MA) calibrated for a monkey eye. For this and all subsequent noninvasive examinations, the animal was tranquilized with intramuscular ketamine HCl (approximately 15 mg/kg). A topical anesthetic (proparacaine HCl 0.5%) was routinely employed when the eye was manipulated.

After these preliminary steps, the basic protocol for each animal was as follows. In the first week, a pigment infusion experiment was carried out. Details of this technique, pigment preparation, and follow-up perfusions are outlined below. Biomicroscopy, sometimes including slit-lamp photographs, and pneumotonometry were performed the next day. Within 5 days after the pigment infusion, goniophotographs of the experimental and control eyes were taken. One week after the initial experiment, a follow-up anterior chamber perfusion was performed. Subsequent follow-up per-
fusion experiments were done at 5, and later 10-wk intervals. Gonioscopy and pneumotonomometry were performed at least every other week.

**Pigment Infusion Experiment**

A primary goal was to cause significant acute obstruction to outflow in a monkey eye using homologous pigment. Early work revealed that large amounts of pigment would be required to accomplish this; essentially the anterior chamber contents would have to be completely exchanged. Another problem was the tendency for pigment to settle rapidly out of suspension. Clumped pigment would block needles, causing flow to cease, thus invalidating pressure measurements. A three-needle technique that featured a servo-perfusion apparatus, a bypass coil that could be loaded with pigment at the last minute, and an exhaust reservoir was adopted.

Anesthesia consisted of intramuscular methohexital sodium (Brevital Sodium, Lilly Research Labs; Indianapolis, IN 15 mg/kg) and intramuscular sodium pentobarbital (35 mg/kg) (Lemmon Co.; Sellersville, PA), as recommended by Kaufman and Barany. Additional pentobarbital was given if needed. After the servo-perfusion syringes, exhaust reservoir, tubing and pressure transducer were filled with mock aqueous (Dulbecco’s PBS with added 5.5 mM glucose) and de-bubbled, the anesthetized animal was placed supine on a contoured restraining device. The first eye to be perfused was given topical proparacaine. The corneas of both eyes were intermittently given methylcellulose 1% topically throughout the experiment to prevent drying.

The first eye to be perfused was alternated between the experimental (pigment) eye and the control eye from one monkey to the next. A control eye was treated identically to an experimental pigment eye except that mock aqueous humor was infused for 8 min in place of pigment. After both eyes had been perfused, subconjunctival (gentamicin sulfate and methicillin sodium) and topical (erythromycin) antibiotics were administered.

**Pigment Preparation**

Four cynomolgus monkey eyes were used to harvest pigment for each pigment infusion experiment according to a technique modified from Shearer. They were received moist, chilled, and fresh from a primate supply house, and were stored at −80°C. Twenty-four or sometimes 48 hr before an infusion experiment, the thawed globes were cleansed with a neomycin sulfate/polymyxin B sulfate solution (Statrol) and finally rinsed with sterile saline. All further steps were carried out under antiseptic conditions.

The anterior third of each eye was separated from the posterior pole. Iris and ciliary body, with as much pigment as possible, were placed in a jar containing 40 ml sterile water. After all four eyes had been dissected, the jar contents were gently mixed and refrigerated.

Prior to the start of a pigment experiment, preliminary centrifugation of the jar fluid at 121 × g for 5 min was carried out to eliminate cellular debris. The
supernatant was respun at \(841\times g\) for 15 min. Pigment pellets that resulted were quickly suspended in one times their volume of mock aqueous humor. Intermittent vortexing kept the final pigment suspension from settling prior to its introduction into the pigment coil.

The homogeneity of this melanosome preparation was confirmed by scanning electron microscopy. A hemocytometer was used to calculate the concentration of pigment particles as well as to confirm the absence of clumping. Using this method, average pigment particle density was determined to be \(1 \times 10^6\) pigment particles per \(\mu\)l.

**Follow-up Perusions**

These experiments required only the pressure and flow needles to be inserted. The basic two-step constant-pressure technique was employed. For a given monkey the eyes were perfused in the same order as in its initial pigment experiment. Anesthesia and other drugs were also similar.

**Morphology**

Animals were deeply anesthetized and both anterior chambers were cannulated. Upon completion of facility measurements eyes were fixed by switching the perfusion fluid to a mixture of 3% glutaraldehyde and 0.1M cacodylate buffer (pH 7.2). This occurred without any interruption of flow and at the normal IOP of 15 mm Hg. Following administration of a lethal dose of anesthetic the eyes were enucleated, opened equatorially and the crystalline lenses were removed. The anterior segment of each eye was immersed in fixative fluid for an additional 2 hr and washed overnight in cold buffer. Subsequently, the anterior segment of each eye was divided into four quadrants; half of each quadrant was processed for light and transmission electron microscopy and half for scanning electron microscopy using our previously described protocol.

**Morphometric studies:** To determine if trabecular cell loss had resulted from either single or multiple pigment infusions, we adopted the morphometric method of Alvarado, et al. Photographic montages were made of one section from each quadrant of each experimental and control eye. The number of nuclei present within the filtration region of each section was counted and the total area of trabecular tissue was determined by tracing the perimeter of the tissue areas using a computer-coupled digitizing planimeter (BOA-10, Bio-Optics, Inc.; Arlington, MA). Data from the four sections of each eye were pooled and the results obtained from experimental and control eyes were compared.

**Results**

In the acute experiments, pigment particle perfusion resulted in a substantial obstruction to aqueous humor outflow. Outflow facility decreased 64% in the experimental pigment perfused eyes, compared to a 76% increase in the sham manipulated fellow eyes, the expected “washout effect.” (One control eye was eliminated from the data due to technical problems—no data is available.) However, 1 wk later when outflow facility was measured again, it had returned to baseline levels in these experimental pigment-perfused eyes (Fig. 1).

Subsequent repeat pigment perfusions in the same experimental eyes produced a similar result: there was acute obstruction to outflow produced by the pigment
particle perfusion, but full recovery of outflow facility subsequently ensued.

At no time was elevated intraocular pressure documented in the pigment perfused eyes (compared to fellow control eyes). Slit-lamp examination performed 24 hr after a perfusion experiment indicated minimal inflammation in both the experimental and control eyes. In experimental eyes, there was a small amount of pigment circulating in the aqueous humor in the anterior chamber and deposited on the corneal endothelium. Gonioscopically a dense well-defined band of pigment was observed in the trabecular meshwork overlying Schlemm’s canal and extending 360° throughout the angle. During the first week after a pigment perfusion, it was usual, especially inferiorly in the angle, to also observe pigment deposited anterior to Schwalbe’s line and on the ciliary body band (which occasionally was quite heavy) and peripheral iris. Nevertheless, it was quite remarkable how the pigment accumulated in a discrete band within the trabecular meshwork. Beginning several weeks after a pigment perfusion experiment, the trabecular pigment band was observed to be decreasing in density of pigment accumulation (Fig. 2), and this continued over the subsequent 3–6 mo.

We are continuing long term observations with follow-up outflow facility measurements in three monkeys, who have had 4, 4, and 1 pigment perfusion experiments, respectively. The last follow-up facility measurement was performed 20, 19, and 33 mo respectively after the last pigment exposure. Outflow facilities, μl/min/mm Hg (and intraocular pressure, mm Hg) in experimental versus control eyes are as follows: 0.28 vs 0.22 (15, 15), 0.32 vs 0.53 (18, 18), and 0.23 vs 0.21 (17.5, 18.5), respectively.

**Morphology**

**Control eyes:** All control eyes appeared essentially normal by both light and electron microscopy. A small amount of pigment was evident within scattered trabecular endothelial cells.

**Experimental eyes:** When the experimental eyes of animals killed one day after either their first or a subsequent pigment infusion were examined, a prominent polymorphonuclear leukocytic (PMN) infiltration was invariably present. Lymphocytes and plasma cells were not routinely observed in any of these animals. The PMN’s appeared to have emigrated primarily from the ciliary body stroma and they occupied the intertrabec-
Fig. 3. Transmission electron micrograph of an animal killed 24 hr after pigment infusion demonstrates polymorphonuclear leukocytes (PMN) in various stages of degranulation and pigment phagocytosis (X5169).

ular spaces and the juxtacanalicular meshwork. By electron microscopy PMN's were identified in various stages of degranulation and pigment phagocytosis (Fig. 3). Trabecular cells had also phagocytosed a variable amount of the pigment, but this was generally less than had been ingested by the PMN's (Fig. 3).

At one week after pigment infusion, scanning electron microscopy (SEM) revealed that a significant number of rounded cells still occupied the intertrabecular spaces, but the trabecular endothelium was largely intact (Fig. 4). Higher magnification revealed that the rounded cells contained large amounts of granular material presumed to be pigment (Figure 4a). Transmission electron microscopy of these same eyes, however, revealed that the cells observed by SEM were not PMN's but instead pigment-laden macrophages and rounded trabecular cells (Fig. 4b).

In long-term experiments (42 and 105 days) the appearance of the trabecular meshwork was surprisingly normal (Fig. 5). Only a small number of pigment-laden macrophages remained within the meshwork. The trabecular endothelial cells contained more pigment than those of control eyes, but their morphological appearance was otherwise similar (Fig. 6).

We had expected that there would be profound trabecular endothelial cell loss as a consequence of pigment phagocytosis and subsequent cellular migration. Despite the infusion of massive amounts of pigment (in some cases three times), however, our initial impression was that profound trabecular endothelial cell loss had not occurred preferentially in experimental eyes. To determine if our impression was correct, a morphometric analysis was undertaken.

Morphometry: Results of the morphometric studies are outlined in Table 1. No greater cell loss was present in experimental compared to control eyes at any stage. Interestingly, the pair that came closest to being significantly different was from the animal killed 24 hr after its first pigment infusion. (Importantly, in animals examined 24 hr after their last pigment infusion, neither the cellular areas of the PMN's nor their nuclei were included in the data collection.)

Discussion

In our study of normal living cynomolgus monkeys we have confirmed that, as in excised eyes, perfusion of pigment particles into the anterior chamber causes a substantial acute obstruction of aqueous humor outflow. However, in these normal, young, animal eyes,
Fig. 4. Scanning electron micrograph of the uveal face of the trabecular meshwork from an animal killed 1 wk after pigment infusion. Note the rounded cells in the intertrabecular spaces (X498). a. At higher magnification these cells are seen to contain large amounts of clumped, granular material (X1608). b. Transmission electron microscopy revealed such cells to be rounded trabecular cells and macrophages which are filled with pigment (X4368).

complete return of outflow facility to normal ensues and pigmentary glaucoma does not develop despite the massive amounts of pigment perfused and the repetitive exposures. Although there are obvious limits to our model system, the results suggest to us that the development of pigmentary glaucoma in humans may require other abnormalities in the trabecular meshwork in addition to the pigment accumulation. This would be consistent with our long-term clinical observations that the full pigmentary dispersion syndrome may occur in normal (usually myopic) human eyes without any abnormality in aqueous humor outflow facility.19,30

Despite the full recovery of outflow function that we demonstrated, it can be questioned whether these animals with longer follow-up would have begun to demonstrate impaired facility of outflow. We have continued to follow three monkeys long-term after pigment perfusion, and with follow-up from 19 to 33 mo, no such abnormality has yet been detected (although one monkey is demonstrating some asymmetry of outflow facility, which is still, however, in the normal range). It can be questioned whether similar results would have been obtained if older rather than young animals had been studied. However, it must be pointed out that the pigmentary dispersion syndrome and pigmentary glaucoma are observed in relatively young adult humans, at an age where a substantial coexistent age-related decrease in outflow facility would not be expected. One might also ask whether we would have observed similar results if a greater number of pigment perfusions had been conducted. Obviously, there are limitations of any model system, but the fact that we perfused much more pigment overall than would be liberated from a single eye's iris and that we observed no chronic impairment in outflow function suggests to us that an otherwise normal trabecular meshwork can fully "process" such liberated pigment without residual abnormality.

How, in fact, is such pigment "processed" within the trabecular meshwork? It was remarkable to us that instead of a diffuse scattering of pigment throughout the angle, a discrete band of pigment overlying Schlemm's canal was observed (Fig. 2). This indicated to us that the pigment moved through the angle along
the route for aqueous humor outflow. Although it is possible that extracellular pigment in the outflow pathway may have been washed out during preparation, the morphological findings indicated that after a week's time, most of the pigment band observed represented pigment phagocytosed by trabecular meshwork cells. We observed gonioscopically that the density of this pigment band decreased progressively over several months.

This phenomenon is sometimes seen clinically in pigmentary dispersion syndrome. The simplest and most widely accepted mechanism to account for this observation is the detaching of pigment-laden trabecular endothelial cells from the beams and their leaving the eye. Therefore, we were surprised to observe a close to, if not totally, normal number of trabecular meshwork cells on the beams in long term experiments (Table 1). (It is also possible that a gradual reduction...
in the number of pigment-laden phagosomes seen in Fig. 4 could account for some of the gonioscopically observed decrease in trabecular pigmentation.)

What do these studies tell us about the "process" of pigment removal from the meshwork? It would seem to us that following the exit of pigment-containing trabecular meshwork cells, either there is replenishment of the meshwork endothelial cells from some source or the pigment is, in fact, removed from the meshwork not by trabecular endothelial cells which migrate from the beams, but by some other type of phagocytic cells, perhaps wandering macrophages. The latter possibility would presumably require some type of transfer of pigment particles from trabecular endothelial cells that were observed initially to have phagocytosed the pigment. A similar conclusion regarding these two possibilities was reached in a study of the long-term phagocytic response of the cat trabecular meshwork to other types of particles. 35

We observed polymorphonuclear cells acutely in the trabecular meshwork after our pigment perfusions. These cells presumably aided in the immediate removal of some pigment material, but they were not present in long-term experiments, and we do not believe they were involved in the long-term decrease in the trabecular pigment band which we observed.

Following the pigment particle perfusion, trabecular meshwork cells and structure appeared normal in long-term experimental eyes. This is contrary to some reports, 36 but is consistent with tissue culture studies 37, 38 where pigment particles were observed to alter neither trabecular cell function nor morphology. We found no evidence to suggest that homologous pigment particles were toxic or antigenic in the trabecular meshwork.

In this regard it is important to note that only a PMN response attended the first and all subsequent pigment perfusions. This suggests that an antigenic sensitization to the homologous pigment and an immunologic response to the potential challenge of later pigment infusions were unlikely. Instead, each administration of pigment appears to have presented simply a phagocytic stimulus to the trabecular meshwork.

Richardson has observed trabecular collapse and other structural abnormalities in surgical specimens from patients with pigmentary glaucoma. 18 Assuming that these morphological findings are not due to drug therapy for glaucoma, our results would suggest that the pigment accumulation, by itself, would not cause such profound structural abnormalities. A similar conclusion was reached by Robin et al in a histologic study of pigment accumulation in the trabecular meshwork following laser iridectomy in the monkey. 39 It would seem to us that other abnormalities in the trabecular meshwork would be required for pigmentary glaucoma to occur. Abnormalities in the cellular response to the pigment would seem to be most important to investigate. The whole question of trabecular meshwork cellular turnover and interaction with wandering phagocytes may be fundamental to this understanding. In addition, it is important to consider possible effects of other substances that may be liberated from the iris pigment epithelium, besides pigment, in the pathogenesis of the pigmentary glaucoma disease process.

Key words: pigmentary glaucoma, trabecular meshwork, melanosome, phagocytosis, aqueous humor outflow

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References