An Evaluation of Novel Vital Dyes for Intraocular Surgery

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PURPOSE. To evaluate systematically the staining characteristics and safety of potential new dyes for intraocular surgery.

METHODS. Six dyes were included in the investigation: light green SF (LGSF) yellowish, E68, bromophenol blue (BBP), Chicago blue (CB), rhodamine 6G, rhodulinblau-basic 3 (RDB-B3). All dyes were dissolved and diluted in a balanced saline saline solution. The light-absorbing properties of each dye were measured at a concentration of 0.05% between 200 and 1000 nm. Staining characteristics were examined by staining lens capsule tissue and epiretinal membranes (ERMs), removed intraoperatively, with dye concentrations of 1.0%, 0.5%, 0.2%, and 0.05%. Enucleated porcine eyes (postmortem time, 9 hours) were also stained. Dye-related toxicity was evaluated by a colorimetric test (MTT) measuring the inhibition of retinal pigment epithelium (RPE) cell proliferation (ARPE-19) and primary human RPE cells, passages 3–6). Cell viability was also quantified based on a two-color fluorescence cell-viability assay. Dyes were investigated in concentrations of 0.2% and 0.02%.

RESULTS. All dyes investigated in this study stained human lens capsules, removed intraoperatively; ERMs, peeled during macular pucker surgery; and enucleated porcine eyes, depending on the concentration applied. The long-wavelength absorption maximum of the dyes was within the range of 527 to 655 nm at concentrations of 0.05%. Rhodamine 6G and RDB-B3 showed adverse effects on ARPE-19 cell proliferation at a concentration of 0.2% and were excluded from further investigation in primary RPE cells. The remaining four dyes showed no toxic effect on ARPE-19 and primary RPE cell proliferation at concentrations of 0.2% and 0.02%. Cell viability was affected by LGSF yellowish (0.2%) and CB (0.2% and 0.02%). Two dyes (E68 and BBP) showed no relevant toxicity in vitro.

CONCLUSIONS. The systematic evaluation of dyes for intraocular use seems mandatory. In this study four dyes were identified with effective staining characteristics, with two of these dyes having no detectable toxic effect on RPE cells in vitro. (Invest Ophthalmol Vis Sci. 2005;46:3315–3322) DOI:10.1167/iovs.04-1142

At present, vital dyes are used to assist ophthalmic surgery, in both the anterior and posterior segments. Especially in macular surgery, dye-assisted vitrectomy allows better intraoperative visualization of the vitreoretinal interface. Two dyes are commonly used in ophthalmic surgery.

Trypan blue was first suggested for use in staining the lens capsule, to assist in capsulorhexis, and for the evaluation of the corneal endothelium of donor tissue before performing penetrating keratoplasty.1–4 It is now also used for staining of epiretinal tissue and the internal limiting membrane (ILM) in macular pucker surgery.5 Although toxic effects have been described in an animal model,6 no dye-related complications have been described in humans.7–10 Nevertheless, carcinogenic and teratogenic properties of trypan blue have been described in animal models11 and long-term side effects are unknown in humans, so far.

Indocyanine green (ICG) has a long history as a diagnostic tool for the imaging of choroidal perfusion during angiography.12–14 Intraocularly, it was used to stain the lens capsule in mature cataract.15 Recently, ICG was the first dye introduced for ILM staining during macular surgery.16,17 However, the known light-absorbing and photo-oxidative properties of ICG had made this dye applicable also for the destruction of tumor cells in vivo16,19 and in vitro20 and for photodynamic therapy at the level of the choriocapillaris in an animal model.21 Although the intravenous application of ICG is still considered very safe,22 concerns of dye-related toxicity when applied intraocularly emerged after several reports on adverse effects observed experimentally23–29 and clinically.30–35 Because there were no in vivo or in vitro studies preceding the use of ICG in macular surgery, this indication represents an off-label use, despite several reports describing no adverse effects of ICG on functional outcome.36–39

 Because of the limitations of trypan blue and ICG, the present study was performed to evaluate the staining characteristics and safety of potential new dyes for intraocular surgery. We tried to establish a systematic approach to potential novel dyes for intraocular surgery. The purpose was to establish a reliable method for evaluating vital dyes before the anticipated application in vivo, be it in animal models or in humans.

MATERIALS AND METHODS

Methods for securing human tissue were humane, included proper consent and approval, complied with the Declaration of Helsinki, and were approved by the local ethics committee. Patients provided informed consent for the epiretinal membranes (ERMs) and lens capsules removed intraoperatively to be used for experimental purposes.
Dye Selection and Evaluation of Staining Characteristics

Six dyes were chosen for the present study: light green SF yellowish (LGSF), E68, bromophenol blue (BPB), Chicago blue (CB), rhodamine G6, and rhodulinblau-basic 3 (RDB-B3). ICG (Pulsion, Munich, Germany) was used as a reference. All dyes were dissolved and diluted in balanced saline (BSS plus; Alcon Laboratories Inc., Fort Worth, TX) and concentrations of 1.0%, 0.5%, 0.2%, and 0.05% were obtained. Dry ICG powder was first dissolved with sterile water provided by the manufacturer, resulting in a 0.5% solution, and then further diluted with balanced saline solution to a concentration of 0.05%. The dyes were then used to stain lens capsules and ERMs removed during intraocular surgery. Immediately after removal, the tissue was placed on a glass slide and covered with a few drops of the dye. After 1 minute, the dye was carefully removed by irrigation with the salt solution. The lens capsule and epiretinal tissue was then evaluated macroscopically and by light microscopy. The staining effect was subjectively graded as excellent, good, fair, or absent by one unmasked coauthor (SGP), and photographs were taken. In addition, we evaluated the staining characteristics of the lens capsule in enucleated porcine eyes with a postmortem time of 9 hours. The dyes were injected into the air-filled anterior chamber and removed by irrigation after 1 minute. Then, the cornea was removed, and a capsulorhexis was performed with a bent needle. All procedures were recorded on video. Rhodamine G6 and RDB-B3 were not tested in this setting, because these dyes showed toxic effects in our cell culture model, as described later.

Light-Absorbing Properties

Light absorption was measured in 0.05% solutions, with balanced saline as a solvent medium. Immediately after preparation of a stock solution of the dye, light absorption was measured with a spectrometer (UV/VIS/NIR Lambda 900; PerkinElmer, Wellesley, MA) between 200 nm and 1000 nm. In comparison with ICG, the solubility of the other dyes in balanced saline is much higher (>0.05% dye).

Evaluation of Dye Toxicity

Human RPE Cell Culture. RPE cells from five human donors were obtained from the eye bank at Ludwig-Maximilians University and were prepared as described previously.40 In brief, whole eyes were thoroughly cleansed in 0.9% NaCl solution, immersed in 5% polyvinyl pyrrolidone iodine, and rinsed again in the sodium-chloride solution. The anterior segment of each donor eye was removed, and the posterior poles were examined with the aid of a binocular stereomicroscope, to confirm the absence of gross retinal disease. Next, the neural retinas were carefully peeled away from the RPE-choroid-sclera, with forceps. The eye cup was rinsed with Ca2+- and Mg2+-free Hanks’ balanced salt solution, and filled with 0.25% trypsin (Invitrogen-Gibco, Karlsruhe, Germany) for 30 minutes at 37°C. The trypsin was carefully aspirated and replaced with Dulbecco’s modified Eagle’s medium (DMEM; Biochrom, Berlin, Germany) supplemented with 20% fetal calf serum (FCS; Biochrom). With a pipette, the medium was gently agitated, releasing the RPE cells into the medium while avoiding damaging Bruch’s membrane. The RPE cell solution was transferred to a 50-mL flask (Falcon, Wiesbaden, Germany) containing 20 mL of DMEM supplemented with 20% FCS and maintained at 37°C and 5% carbon dioxide. Epithelial origin was confirmed by immunohistochemical staining for cytokeratin with a pan-cytokeratin antibody (Sigma-Aldrich, Deisenhofen, Germany).15 The cells were tested and found to be free of contaminating macrophages (anti-CD11; Sigma-Aldrich) and endothelial cells (anti-von Willebrand factor; Sigma-Aldrich; data not shown). After having grown to confluence (100%), primary RPE cells were subcultured and maintained in DMEM supplemented with 10% FCS at 37°C and 5% carbon dioxide. Primary RPE of passages 3 to 6 and ARPE-19 cells were used in the experiments.

ARPE-19 cells, a human RPE cell line, were purchased from American Type Culture Collection (Manassas, VA), maintained in a 1:1 mixture of DMEM and Ham’s F12 medium (DMEM/Ham’s F12; Biochrom), supplemented with 10% FCS.

MTT Assay. For exposure of the dye, the RPE and ARPE-19 cells were kept for 24 hours in serum-free conditions. After the cells were washed three times with PBS, they were incubated for 10 minutes with 300 μL of balanced saline containing 0.2% or 0.02% of dye, respectively. This rather long exposure time seemed reasonable to us, as it increases the chance of detecting toxicity, but does not mimic the current clinical use of ICG or trypan blue or the likely use of any new dye. The dye was then removed by carefully rinsing cells with balanced saline three times. After 24 hours of incubation with serum-containing medium the cell proliferation assay was performed. RPE cells (passages 3–6) were seeded in 24-well plates and exposed to two concentrations (0.2% and 0.02%) of dyes. Balanced saline alone and H2O2 (200 μL/mL) served as the control.

The tetrazolium dye-reduction assay (MTT; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was used to determine the cell survival rate. The MTT test was performed as described in the literature by Mosmann,43 with some modifications. The medium was removed, cells were washed with PBS, and 1000 μL/well MTT solution (1.5 mL MTT stock, 2 mg/mL in PBS, plus 28.5 mL DMEM) was added. RPE cells were incubated at 37°C for 1 hour. The formazan crystals that formed were dissolved by the addition of dimethylsulfoxide (DMSO; 1000 μL/well). Absorption was measured by a scanning multiwell spectrophotometer at 550 nm (Molecular Probes, Garching, Germany). Results were expressed as the mean percentage of control proliferation. Experiments were performed in triplicate and repeated three times. RPE and ARPE-19 cells of the same passage incubated with balanced saline without the addition of dyes served as the control. Statistical comparison between dye concentrations was performed on computer (SPSS software; SPSS Science, Chicago, IL; Mann-Whitney test).

The MTT test, as performed in our study, is a well-established test for the assessment of cell viability, but relies on colorimetric measurement of a blue (550 nm) formazan reaction product. This color overlaps the absorption spectra of some of the dyes tested. Therefore, we performed control experiments to check for potential interference of residual dye with the assay. Cell monolayers were treated with dyes as per our experiments described earlier, but absorbance readings were performed without prior application of MTT. We found no differences after dye treatment compared with the balanced saline control. Experiments were performed in triplicate and repeated three times.

Live–Dead Cell-Viability Assay. Confluent RPE and ARPE-19 cells were prepared and treated as described for the MTT assay. Cell viability was quantified based on a two-color fluorescence assay in which the nuclei of nonviable cells appear red because of staining by the membrane-impermeable dye propidium iodide (Sigma-Aldrich), whereas the nuclei of all cells were stained with the membrane-permeable dye Hoechst 33342 (Intergen, Purchase, NY). Confluent cultures of RPE cells growing on coverslips in 24-well tissue culture plates were exposed to 0.2% and 0.02% LGSF yellowish, E68, BPB, and CB as described for the MTT assay. For evaluation of cell viability, the cells were washed in PBS and incubated with 2.0 μg/mL propidium iodide and 1.0 μg/mL Hoechst 33342 for 20 minutes at 37°C. Subsequently, the cells were analyzed with an epifluorescence microscope (Axioskop; Carl Zeiss Meditec, Inc., Göttingen, Germany). The labeled nuclei were then counted in fluorescence photomicrographs, and dead cells were expressed as a percentage of the total nuclei in the field. The data are based on counts in three experiments performed in duplicate wells, with three to five documented representative fields per well. RPE and ARPE-19 cells of the same passage incubated with balanced saline without the addition of dyes and H2O2 (200 μL/mL) served as the control.
RESULTS

Evaluation of Staining Characteristics

The staining effect in surgically removed lens capsules and ERM varied between the different dyes and dye concentrations was determined (Figs. 1, 2). LGSF yellowish did not stain lens capsules sufficiently at concentrations of 0.5% or less, and only a concentration of 1% provided weak staining of ERMs. Lower concentrations of LGSF therefore were not tested. The other dyes showed excellent to good staining effects, both in lens capsules and ERMs, even at a lower concentration of 0.2%. The grading of the staining effect of each dye and dye concentration is shown in Table 1. In porcine eyes, the lens capsule stained well with BPB, CB, and E68, whereas LGSF yellowish stained the tissue weakly (Fig. 3). In general, the contrast in porcine eyes was less pronounced, as the staining effect was evaluated against the background of the clear porcine lens. During surgery in humans, dyes are used to assist capsulorrhexis predominantly in mature cataracts, where one would expect a much better contrast.

Light-Absorbing Properties

The light-absorbing properties and peaks of maximum absorption of dye concentrations of 0.05% were variable. The long-wavelength maximum peak of absorption was in the range of 527 to 655 nm (Fig. 4). Except for LGSF yellowish and rhodamine 6G, no dye showed relevant light absorption between 400 and 500 nm. Absorption maxima beyond 700 nm were not found with any of the investigated dyes.

Evaluation of Dye Toxicity

MTT Assay. Compared with the balanced-saline control, four novel dyes—LGSF yellowish, E68, BPB, and CB—had no significant impact on the survival of ARPE-19 cells either concentration (0.2% or 0.02%). Rhodamine G6 and RDB-B3 revealed toxic effects in a concentration of 0.2%, which was less...
RESULTS

The staining effects were graded as excellent (+ + +), good (+ +), fair (+), and absent (−). Yellowish LGSF, E68, BPB, CB, Rhodamine G6, and RDB-B3. The staining effect was evaluated against a white background. Symbols in parentheses indicate an “in between” grading—that the staining was less pronounced.

severe in a concentration of 0.02% (Fig. 5). In addition, no influence on the survival of primary RPE cells was observed after exposure to LGSF, E68, BPB, and CB at concentrations of 0.2% and 0.02% (Fig. 6). The differences between both concentrations were statistically significant only with rhodamine G6 (P ≤ 0.05) in ARPE-19 cells and with LGSF yellowish (P ≤ 0.05) and ICG (P ≤ 0.05) in primary RPE cells.

DISCUSSION

The use of vital dyes intraoperatively to stain ocular tissue such as the lens capsule, ERM, or ILM potentially facilitates ophthalmic surgery. The introduction, especially of ICG to assist macular surgery was initially met with great enthusiasm in the ophthalmology community, as it appeared to make intraocular surgery safer and more controllable by enabling visualization and facilitating the removal of the ILM during surgery for traction maculopathies, such as macular pucker or macular hole. In addition, staining of the vitreoretinal interface could open the door for the less experienced surgeon to follow the principle of ILM removal in macular surgery. However, as there have been observations indicating potential dye-related toxicity in circumstances not yet completely understood, the use of ICG has become a controversial subject among surgeons. The question of whether ICG should be considered a “toxic adjunct” is currently under investigation. No in vivo or in vitro safety studies concerning the intraocular use of ICG preceded the clinical intraocular application of ICG. It thus appeared reasonable to us to search for novel dyes that are safe for intraocular application and offer a potential alternative to ICG in the future. Therefore, we performed several screening
tests to ensure efficacy and safety of these novel dyes before attempting in vivo application.

Given the known chemical properties of ICG and the current debate on its potential toxic effects after intraocular application, the following considerations might be of interest when choosing and evaluating novel dyes:

1. The dye should have a large absorption coefficient. Large extinction coefficients allow for the injection of a significantly lower amount of the dye. As a consequence, the formation of aggregates is suppressed.

2. The dye should have high solubility in water and balanced saline (which is used as an irrigation solution during surgery), but solubility should not be concentration dependent. In most cases, this effect is accompanied by a dramatic shift of the absorption maximum and/or by the appearance of new absorption bands.

3. The material should have a high photochemical stability. That means that an irreversible photoreaction in the first excited singlet state (S1) or triplet state (T1), as described for the photosensitizer ICG, should be avoided.

**Figure 4.** The light-absorbing properties of LGSF yellowish, E68, BPB, CB, rhodamine G6, and RDB-B3. The maximum peak absorption varied between 527 to 655 nm. Most dyes showed no relevant light absorption between 400 and 500 nm or beyond 700 nm.
4. The triplet quantum yield should be as low as possible, avoiding singlet oxygen formation that can lead to irreversible oxidation of the ILM or other tissue or to photodegradation of the dye itself. In contrast, the life of singlet oxygen both in water and in the solid state is very short.

5. The staining material should have minimal dark toxicity.

6. The dye should exhibit very good adsorption properties toward the target tissue.

It is evident from this brief enumeration that all requirements can hardly be met, and compromises should be found. We selected different dyes of both cationic and anionic character from different dye classes. These groups generally have large absorption coefficients ($5 \times 10^4 - 2 \times 10^5$ L mol$^{-1}$ cm$^{-1}$). All compounds show a high solubility in water and balanced saline. Their photostability and dark stability in water or balanced saline are in some cases much better than those of ICG (data not shown). Two of them, rhodamine 6G and RDB-B3, showed marked fluorescence (fluorescence quantum yield, $>0.7$). The position of the long-wavelength maxima of LGSF yellowish, E68, BPB, CB, rhodamine 6G, and RDB-B3 were not influenced by the dye concentration (measured up to 0.05% dye).

As we hypothesized that dyes staining the lens capsule, which represents a basement membrane, would also sufficiently stain the ILM, the basement membrane of the retina, we incorporated dyes in this investigation that only provided sufficient staining of the lens capsule. Of note, vital stains currently used in ophthalmic surgery, such as trypan blue and ICG, stain both the lens capsule and the ERM or ILM, respectively.4,5,15,16 Therefore, only the dyes providing effective staining, as seen with the six dyes in the present investigation, were considered for the in vitro experiments using ARPE-19 and primary RPE cells. However, with the in vitro approach presented in this study, we cannot exactly predict how these
dyes would bind to ocular tissue in vivo. It may be that the staining characteristics of a dye would be altered when staining tissue with damaged surfaces as may be the case with the excised lens capsule. Therefore, we also performed staining experiments in enucleated porcine eyes, in which the dye could be delivered to the undamaged surface of the lens capsule. A similar approach to finding an appropriate control to test ERMs was impossible.

In the present study in vitro investigations were performed with both semiconfluent ARPE-19 cells and primary RPE cells treated with dye concentrations of 0.2% and 0.02%. The rationale for this approach was on the one hand that proliferating cells are more sensitive toward toxic agents than nonproliferating cells. On the other hand, previous studies evaluating the effects of ICG,23,24,45,46 or trypan blue25 on RPE cell cultures were almost exclusively performed in only one RPE cell line, either using ARPE-19 or primary RPE cells. In addition, clinical effects of ICG23,24,45,46 or trypan blue45 on RPE cell cultures were almost exclusively performed in only one RPE cell line, either using ARPE-19 or primary RPE cells. In addition, clinical reports have suggested an adverse effect of ICG on human RPE cells. Other cell types than RPE cells, such as retinal glial or ganglion cells, have been rarely used.46 The strength of the present study was that the effect of the dyes was investigated in two RPE cultures, ARPE-19 and primary RPE cells. Dyes affecting the survival of ARPE-19 cells, as seen after incubation with rhodamine G6 and RDB-3B, were not further evaluated in primary RPE cell lines. In addition, such dyes appear not to be applicable in vivo and will therefore also be excluded from future in vivo investigations in animals. Another strength of our study is that we performed two tests to assess cell viability: the MTT and live–dead cell viability assays. For in vivo investigations, we will now focus on those dyes that showed no impact on the survival of the primary RPE cells.

In summary, in light of the potential toxicity of ICG, the search for new dyes with improved chemical, physical, and spectral properties remains an important goal. In the present study, four dyes showed satisfying staining characteristics, with two of them having no toxic effects on cultured RPE cells in both concentrations tested (0.2% and 0.02%). It is not yet clear whether the dyes investigated in the present study will provide sufficient and effective staining in vivo. Therefore, we currently do not recommend these dyes for clinical application. This study had certain weaknesses, such as the subjective evaluation of the staining effect or the intraoperative setting in porcine eyes with clear lenses, limiting the contrast achieved by lens capsule staining. Nevertheless, some of the dyes evaluated showed promising staining characteristics and safety ex vivo. Another weakness of an ex vivo approach as presented in this report is that it does not, and cannot, replicate the clinical in vivo situation. However, in vitro studies using cell cultures allow us to determine toxicity and safety margins of a new agent. We suggest that a systematic evaluation of novel dyes should be performed before any use in vivo, be it in animal models or in humans. With this step-by-step procedure, we will be able to evaluate thoroughly the risks and benefits of potential novel dyes for ocular surgery.

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References


