Comparative Toxicology of Trypan Blue, Brilliant Blue G, and Their Combination Together with Polyethylene Glycol on Human Pigment Epithelial Cells

Doaa Awad,1,2 Imke Schrader,1 Melinda Bartok,1 Andreas Mohr,3,4 and Detlef Gabel1,4

PURPOSE. To determine the toxicity in ARPE-19 human retinal pigment epithelium cells of trypan blue (TB) at 0.15% and 0.25% concentration, brilliant blue G (BBG) at 0.025% and 0.05%, their combination, and the effect of the addition of 4% polyethylene glycol (PEG), as an additive for increasing the density and thus improving the staining in internal limiting membrane removal, on the individual dyes and their combinations, and compare the toxicity of the dyes to that of clinically used preparations.

METHODS. Cells were exposed for 5 and for 30 minutes to the different preparations. Cell viability was measured with the WST-1 assay measuring intracellular dehydrogenase activity.

RESULTS. Solutions containing PEG with BBG (0.025%), TB (0.15%), and mixtures of BBG (0.025%) with TB (0.15% and 0.25%) were the least toxic of the preparations as well as preparations of BBG at 0.025% in phosphate-buffered saline solution, while TB at 0.25% in phosphate-buffered saline solution was the most toxic. The addition of PEG reduced the toxicity of preparations containing TB either alone or in combination with BBG. These results were seen only after an incubation for 30 minutes; for a 5-minute incubation, no toxicity was seen for any of the preparations.

CONCLUSIONS. For short incubation times, all dyes appear equally safe. For longer incubation times, TB preparations were more toxic than BBG preparations. The toxicity of TB was reduced by the addition of PEG. Further studies are required to determine the clinical impact of this finding. (Invest Ophthal Vis Sci. 2011;52:4085–4090) DOI:10.1167/iovs.10-6336

The removal of the internal limiting membrane (ILM) is a surgical procedure that is used widely in the treatment of macular pathology, e.g., macular hole formation or macular edema due to inflammatory or vascular diseases (for a review, see Ref. 1). The membrane, which is a proteinaceous layer formed by the foot plates of the Müller cells, is difficult to see without staining; complete removal of the ILM during surgery is necessary. Complications, caused by surgical trauma during intervention, can be largely avoided when the ILM is stained before removal. In addition, the completeness of the removal of ILM around the rim of the macular holes can be checked, and the closure rate is thus increased. For a review of the procedure and staining conditions used at present, see Refs. 2–4.

Clinically used stains for this procedure are, among others, trypan blue (TB) and brilliant blue G (BBG).7 Indocyanine green (ICG) is also widely used, whereas other dyes (Infracyanine green, Patent blue, Evans blue) have found less application. Toxicity in cell culture has been reported when these dyes are used at higher concentrations and with longer incubation times. The most recent publication on this subject6 summarizes the toxicity levels associated with commonly used dyes.

The use of several dyes in the same surgical procedure appears not to be widespread. Two dyes, TB and ICG, have been used in sequence in macular pucker surgery,7 where the epiretinal membrane (ERM) was first identified with TB, and subsequently the ILM with ICG. Recently, it has been suggested by one of us (AM at Innovations in Ophthalmology, Kyoto, Japan, 2007) that a combination of TB and BBG administered simultaneously might be even more advantageous for visualizing the ILM as well as ERMs, since both tissues can occur in the same eye simultaneously. The clinical use of this combination requires, however, that the combination of the two dyes at clinically relevant concentrations is not more toxic than the individual dyes.

Generally, the dye preparations presently in clinical use are made up in phosphate-buffered saline solution (PBS). This solution has the same density as the PBS, which is used for restoring the vitreous cavity after the removal of the vitreous body, and therefore it is rapidly dispersed during the surgical procedure. It would, however, be desirable that the dye solution should have both a higher density and a higher viscosity, so that it would initially, after administration, settle on the ERM or ILM before it is removed by passive aspiration with a flute needle. The addition of glucose has previously been suggested.8 As glucose has a strong effect on the osmolarity of the solution, other, high-molecular weight additives are to be preferred. We therefore tested the toxicity of a polyethylene glycol (PEG) solution alone and in combination with the TB and BBG dyes. PEG also leads to a higher viscosity of the injection solution.

We tested the individual dyes, in clinically relevant concentrations, and their combination for cell toxicity. The dyes were dissolved in PBS as well as in PBS containing PEG at 4% (w/w). Toxicity was assessed in cell culture on the human retinal pigment epithelium (RPE) cell line ARPE-19. Incubation times of 5 and of 30 minutes were chosen. These times bracket the realistically achievable contact times in the hands of experienced and of less experienced surgeons. Cell viability was measured using the WST-1 assay, which measures intracellular dehydrogenase activity. This assay is similar to the MTT assay but has the advantage that the colored product is soluble, thus avoiding additional washing and addition of solubilizing agents.
Methods

Dye Solutions

Dyes were prepared for the Dutch Ophthalmic Research Center (Zuidland, The Netherlands) and provided by the Netherlands Institute for Investigative Ocular Surgery (Rotterdam, The Netherlands) and are listed in Table 1. A total of 13 dye preparations were tested. The average molecular weight of the PEG used in the preparations was 3350, and its concentration was 4% (w/w).

The buffer PBSPEG was the buffer used for preparing the dye solutions containing PEG, and the buffer PBS was used for preparing the remaining solutions.

Cell Culture

ARPE-19 cells were obtained from ATCC (cell line CRL-2302; ATCC, LGC Standards GmbH, Wesel, Germany) and grown in Dulbecco’s modified Eagle’s medium (DEME) -F12 medium with 10% fetal bovine serum at 37°C and 5% CO2.

Cell Toxicity Assay

All dyes were tested for acute toxicity in ARPE cells using the WST-1 assay (Roche, CITY, COUNTRY). The WST-1 assay produces a red, water-soluble dye from the formazan precursor by mitochondrial dehydrogenases. It thus resembles the MTT assay but does not require additional washing steps and dissolution of the dye.

For the WST-1 assay, cells were seeded in 96-well plates (20,000 cells per well) and grown for 2 days. The medium was removed by suction, and 50 µL of each dye solution (warmed to 37°C) was added to the wells. After 5 or 30 minutes incubation at 37°C in the incubator, the dye solutions were removed by suction, each well was washed 3 times with PBS, and 100 µL of WST-1 (Roche), diluted in growth medium 1:10, was added to each well. After incubation for 90 minutes at 37°C, the absorbance of the plate was measured at 450 nm in a plate reader (MR8000; Dynatech, CITY, COUNTRY). It was verified that the increase in absorbance for the period of 90 minutes was linear with time, and that the absorbance after 90 minutes is thus a correct representation of the cellular enzyme activity. It was also ensured, by reading plates that have been washed, but to which no WST-1 was added, that any possibly remaining dye did not lead to any increased absorbance at 450 nm. Optically, no dye was visible on the washed plates.

Table 1. List of Solutions Tested

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Solution Description*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB0.15</td>
<td>TB 0.15%</td>
</tr>
<tr>
<td>TB0.25</td>
<td>TB 0.25%</td>
</tr>
<tr>
<td>BBG0.025</td>
<td>BBG 0.025%</td>
</tr>
<tr>
<td>BBG0.05</td>
<td>BBG 0.05%</td>
</tr>
<tr>
<td>Monoblue</td>
<td>MonoBlue TB 0.25% (Arcadophtha; 9423054)</td>
</tr>
<tr>
<td>BrilliantPeel</td>
<td>Brilliant Pecl, BBG 0.025% (Fluoron; BP130809)</td>
</tr>
<tr>
<td>MembraneBlue</td>
<td>MembraneBlue TB 0.15% (DORC; 31508)</td>
</tr>
<tr>
<td>BBG0.025PEG</td>
<td>BBG 0.025% + PEG-3350 4%†</td>
</tr>
<tr>
<td>BBG0.05PEG</td>
<td>BBG 0.05% + PEG-3350 4%</td>
</tr>
<tr>
<td>TB0.15PEG</td>
<td>TB 0.15% + PEG-3350 4%</td>
</tr>
<tr>
<td>TB0.25PEG</td>
<td>TB 0.25% + PEG-3350 4%</td>
</tr>
<tr>
<td>TB0.15BBG0.025PEG</td>
<td>TB 0.15% + BBG 0.025% + PEG-3350 4%†</td>
</tr>
<tr>
<td>TB0.25BBG0.025PEG</td>
<td>TB 0.25% + BBG 0.025% + PEG-3350 4%†</td>
</tr>
<tr>
<td>PBSPEG</td>
<td>PBS with PEG-3350 4%</td>
</tr>
<tr>
<td>PBS</td>
<td>PBS</td>
</tr>
</tbody>
</table>

* For commercially available preparations, the company and the lot number are given in parentheses. Concentrations (weight/volume) are given after the dye.
† BBG 0.025% + PEG-3350 4% is now commercially available (DORC) as ILM Blue and TB 0.15% + BBG 0.025% + PEG-3350 4% as MembraneBlue-Dual. DORC, Dutch Ophthalmic Research Center.

Cells were incubated with the dyes for 5 or for 30 minutes to determine the influence of the incubation time. Five minutes is the maximum incubation time when the removal of the ILM is executed by an experienced ophthalmologist, and 30 minutes is beyond the time that is conceivable for this surgical procedure.

For each sample on each plate, 12 or 18 wells were used, and the results were averaged. Three or four independent measurements on different days for each dye solution were performed. The value for the cell viability is the average of the independent measurements.

As control, the absorbance of the WST-1 assay of cells incubated with buffer (PBS with PEG for the dye solutions with PEG, and PBS without PEG for the other dye solutions including the commercial preparations) was used.

The results are expressed as the fraction of cells that are viable. A 95% confidence interval was used for statistical evaluation. The 95% confidence interval for cells incubated with the appropriate buffer (PBSPEG or PBS), which was set to 100%, was between 4% and 9% for repetitive experiments. The values for cell survival and confidence intervals for 5 and 30 minutes of incubation are found in Figure 1. The cell viability and the 95% confidence interval after an incubation time of 30 minutes are also given in Table 2.

Statistical Evaluation

Statistical analysis was performed with Microsoft Excel, using the procedure “2-sample t-test with different variances.” Pairs of data were compared. The P values for the different pairs compared are listed in Table 2.

Thin Layer Chromatography

Thin layer chromatography (TLC) was carried out on Polygram Sil G/UV254 TLC plates (size 40 x 80 mm) (Macherey-Nagel, CITY, COUNTRY). The dyes were applied undiluted, and the plate was dried with warm air before development. Development was in a mixture of 70 mL isopropanol and 30 mL concentrated aqueous ammonia solution.

Results

The fraction of viable cells and the 95% confidence interval of the viability after incubation with the different solutions and the two different times are shown in Figure 1. The viability of the appropriate control (PBS for solutions prepared in PBS, PBS + PEG for solutions containing PEG) was set to 100%.

Toxicity of Buffers

The two PBS solutions used showed a reduced viability when compared with cells incubated with growth medium. The viability for PBS + PEG was 92 ± 6% (95% confidence interval) after 5 minutes incubation, and 84 ± 6% after 30 minutes incubation. For PBS, the corresponding values for 5 and 30 minutes incubation were 81 ± 3% and 82 ± 5%, which at 5 minutes incubation is significantly lower (P ≤ 0.05) than in the presence of PEG.

Toxicity of Dyes

After very short incubation times (15 seconds, the time required for adding the dye and removing it again by aspiration), cells incubated with any of the dye solutions had a higher response in the WST-1 assay than those incubated with the corresponding buffer PBS or PBSPEG alone. The increase was by a factor of 1.24 ± 0.08.

After incubation for 5 minutes, the viability of the cells, as determined by the WST assay, was between 100% and 114% relative to the control with the corresponding PBS or PBSPEG buffer. As indicated in Figure 1, none of the preparations with a single dye was more damaging than the control. For an incubation time of 30 minutes, viability varied between 114%
and 79%. Brilliant Peel was less damaging than the corresponding buffer, whereas BBG0.05, TB0.25, and MonoBlue were more toxic (Fig. 1).

With an incubation time of 5 minutes, no statistically significant cell toxicity was found when each of the possible pairs of dyes were compared. After 30 minutes of incubation, statistically significant ($P < 0.05$) differences in viability between several of the dye preparations were found when compared to the same dye after 5 minutes exposure (Fig. 1). When compared pairwise, significant differences in toxicity were found for some of the pairs. These pairs can be found in Table 2, together with the $P$ value. As expected, TB0.15 is less toxic than TB0.25, and BBG0.025 is less toxic than BBG0.05.

Of the commercial preparations, MonoBlue (TB0.25%) is the most toxic preparation and therefore more toxic than MembraneBlue (TB0.15%) and even more toxic than Brilliant Peel (BBG0.025%), but this was visible only after an incubation time of 30 minutes. The observed toxicity difference between MonoBlue and MembraneBlue is not surprising, as MonoBlue contains TB at a higher concentration than MembraneBlue.

When the commercial preparations are compared with a separately prepared dye solution (MonoBlue versus TB0.25, MembraneBlue versus TB0.15, Brilliant Peel versus BBG0.025), no difference in toxicity was found within any of the three pairs, thus mutually confirming the toxicity results for these combinations.

We found that of the single dye preparations, BBG0.025%, which contains the lowest dye concentration of all preparations, was the least toxic of the preparations tested, whereas TB0.25 was the most toxic of these preparations; toxicity was visible, however, only after 30 minutes incubation, and not after 5 minutes incubation.

Influence of PEG

The addition of PEG to dyes did not lead to an increase in toxicity in any case where the same dyes could be compared (BBG0.025PEG and BBG0.025, BBG0.05PEG and BBG0.05, TB0.15PEG and TB0.15, TB0.25PEG and TB0.25). While the TB pair TB0.15PEG and TB0.15 did not show significantly different toxicity, this was changed for TB0.25PEG and TB0.25. In this case, PEG reduced the toxicity associated with TB, reducing the toxicity of TB0.25PEG to that of TB0.15. The preparation TB0.15PEG was one of the six preparations with the lowest toxicity.

Influence of Combination of Dyes

Combinations of dyes were tested only in the presence of PEG. The combination of dyes in the presence of PEG led, unexpectedly, to a reduction of toxicity. None of the two combinations (TB0.15BBG0.025PEG and TB0.25BBG0.025PEG) led to a further decrease in viability when compared with TB0.15PEG, TB0.25PEG and BBG0.025PEG. Thus, the toxicity of the dyes (in the presence of PEG) cannot be regarded as additive. Rather, the statistically significant toxicity of TB in a concentration of 0.25% (TB0.25PEG) is completely neutralized by the addition of BBG at 0.025% (TB0.25BBG0.025PEG). This is surprising, as one would have expected the combination of TB and BBG to be at least as toxic as the same concentration of TB alone.

Comparison between 5 and 30 Minutes Incubation

When comparing the effect of identical preparations after an incubation for 5 minutes with incubation for 30 minutes, the
samples TB0.25, MonoBlue, and BBG0.05PEG were more toxic (marked with an asterisk in Fig. 1). For all other preparations, toxicity after incubation with the dye for 30 minutes was not higher than after an incubation for 5 minutes, when a level of significance of $P \leq 0.05$ is considered.

### Purity of Dyes

Figure 2 shows that the dye solutions of TB and BBG (MembraneBlue and Brilliant Peel) are not made up from pure chemical compounds but contain a number of colored compounds in addition to the main band. For TB, a pink component with an $R_f$ value of 0.55, moving faster than the main component, could be seen in all preparations. The relative amounts of impurities varied between the different sources of the solution. Purple fractions have been found before in TB$^9$ and have been identified as monoazo dyes.

### Discussion

Potential cell toxicity of dyes in intraocular surgery is an unwanted feature. When choosing an assay system to assess toxicity, the most relevant cells would be those that are in direct contact with the dye solution. Thus it would be desirable to perform toxicity tests on Müller cells, which are the most important cell type for the structure of the retina. Unfortunately, such cells are not readily available$^{10}$ and can be isolated only as primary culture (see, e.g., Ref. 11). The only relevant human cell type available commercially is an RPE cell line. This cell line was chosen, as the preparation of primary cultures would have induced a considerable degree of variation into the results. ARPE cells have also been used to evaluate the toxicity of dyes$^{10,12}$ and other agents.$^{13-15}$

Short incubation (5 minutes) of the cells with the different dye solutions were without effect on the cells, confirming the safety of the commercial dye solutions and of the preparations investigated here. When the results from longer incubation times are considered relevant, BBG at 0.025% is the least cell toxic preparation of the PEG-free preparations. Both preparations of TB at 0.15% are more toxic than BBG at 0.025% when exposure was for 30 minutes. Yuen et al.$^{11}$ have found TB at 0.015% to be less toxic than BBG at 0.025%; the TB concentrations used in their study were, however, considerably smaller, by around a factor of 10, than that of the commercially available solutions and the concentrations of TB used here. Thin layer chromatography (Fig. 2) reveals that the commercial dye solutions contain a number of side products, which indicate that TB consists of more compounds than the claimed structure. Some of these have previously been reported to be oncogenic, whereas the major blue component of TB was found to be teratogenic.$^9$ Toxicity for these purified components has not been investigated. It might well be that differences in toxicity reported by different groups could be caused by different degrees of purity of the dyes; generally, it appears that a purification of the dyes might be advantageous and desirable.

Narayanan et al.$^{16}$ found a statistically significant reduction of cell viability for R28 cells when exposed to TB at a

### Table 2. Cell Viabilities after an Incubation Time of 30 Minutes and $P$ Values of Two-Sample $t$-Tests of Solutions Tested

<table>
<thead>
<tr>
<th>Cell Viability</th>
<th>TB0.25BBG0.025PEG</th>
<th>TB0.15BBG0.025PEG</th>
<th>Brilliant Peel, BBG0.025 (Fluoron)</th>
<th>BBG0.025PEG</th>
<th>BBG0.025</th>
<th>TB0.25PEG</th>
<th>Brilliant Peel</th>
<th>BBG0.025PEG</th>
<th>BBG0.025PEG</th>
<th>BBG0.05PEG</th>
<th>BBG0.05PEG</th>
<th>MembraneBlue TB0.15 (DORC)</th>
<th>TB0.15 (DORC)</th>
<th>TB0.25 (DORC)</th>
<th>MonoBlue TB0.25 (Arcadophta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>95% Confidence Interval</td>
<td>1.137 0.082</td>
<td>1.133 0.083</td>
<td>1.084 0.031</td>
<td>1.083 0.050</td>
<td>1.039 0.096</td>
<td>1.034 0.140</td>
<td>0.953 0.033</td>
<td>0.945 0.019</td>
<td>0.942 0.076</td>
<td>0.900 0.079</td>
<td>0.894 0.056</td>
<td>0.804 0.013</td>
<td>0.792 0.083</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Only values with $P \leq 0.05$ are listed; those having $P > 0.05$ are indicated as ns.

DORC, Dutch Ophthalmic Research Center.

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cells might have divided during this time and might have replaced dead cells.

In conclusion, when short incubation times are considered as relevant for the decision of which dyes are to be considered more or less toxic, none of the different preparations has a definite advantage or disadvantage. This finding is, however, dependent on the amount of dye solution remaining on the retinal surface. Although it can be assumed that all visible dye is washed out, it remains unclear how much of the dyes is bound to other structures within the eye and therefore could be potentially harmful. Especially for beginners in vitreoretinal surgery, who usually require longer handling times and may apply the dye several times, the results found here should be taken with caution. For longer incubation times, TB preparations are more toxic. Combinations of TB and BBG carry no more risk of cell toxicity than any of the two individual dyes.

The addition of PEG reduces markedly the toxicity of TB. Therefore, its inclusion into the formulations for staining might be beneficial not only for the wanted effect of a denser and more viscous solution, but also because of further reduced toxicity of the dye. For BBG as a single-dye component, this protective effect of PEG was not measurable.

**Acknowledgments**

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**References**

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