Objective evaluation of experimental ocular irritation. Eugenio Maul and Marvin L. Sears.

After topically administered nitrogen mustard the irritative effect on the pupil, intraocular pressure, protein concentration of the aqueous humor and on the vessels of the iris and conjunctiva was quantified. The protein level in aqueous was determined by refractometry. The degree of hyperemia of conjunctiva and iris was determined by measuring the amount of Evans blue dye present in the tissue minutes after intravenous administration. A reproducible relationship was obtained using these methods to evaluate the degree of irritation produced by nitrogen mustard.

Nitrogen mustard (NM) applied topically to the rabbit eye causes hyperemia of the iris and conjunctival vessels, miosis, increase in intraocular pressure, and breakdown of the blood-aqueous barrier. For analysis of the mechanism and pathway of this chemical irritation, more precise methods are required to quantify the clinical response known as "irritation." Previous studies of the ocular effect of NM have been done in a qualitative or semiquantitative way.

Materials and methods. Male New Zealand albino rabbits, 2 to 3 kilograms, gently restrained, and lightly anesthetized with sodium pentobarbital 15 mg. per kilogram were used. Nitrogen mustard (NM) solutions (pH 3.0-4.0) in 0.15 M NaCl at 0.1 per cent, 0.5 per cent, 1 per cent, and 10 per cent (W/v) were prepared. Two drops of the irritant were applied to one eye and two drops of saline to the contralateral eye as a control. The chemical was allowed to act for 30 minutes, after which a maximal effect is observed. Under controlled laboratory illumination, the pupil diameter was recorded in millimeters at different times after instillation of drops.

Intraocular pressure was recorded with a pneumatic tonometer calibrated against water, at the beginning and end period of the experiment. At the end of the experiment aqueous humor was obtained through a corneal paracentesis under a Zeiss operating microscope. The protein content was determined by refractometry with the Goldberg temperature compensated refractometer (American Optical). The measurement was done on a 0.05 sample, though only 0.03 ml. are required. The index of refraction obtained for the test sample was extrapolated to a standard curve of 50 to 4,000 mg. per cent of bovine albumin in saline. This method was checked with the method of Lowry and co-workers.

Hyperemia of the conjunctiva and the iris was determined by assaying the amount of Evans blue present in the tissue. Thirty minutes after instillation of NM, 50 mg. per kilogram of a 50 mg. per milliliter Evans blue solution in distilled water was injected into the marginal vein of the ear. After five minutes the animal was sacrificed with gallamine triethiodide and potassium chloride. The rabbits were dead with instantaneous arrest of circulation and without convulsions. The conjunctiva was quickly excised and after removal of the cornea the complete iris was obtained. The wet weight of the tissues was determined, and then cut into small pieces. Dye was extracted by adding 2 ml. of 0.15 per cent NaHSO3 in 70 per cent acetone (extraction reagent). The tube containing the tissue plus extraction reagent was set into an ultrasonic bath for 30 minutes, then centrifuged for 20 minutes at 2,000 r.p.m. The absorbance of the supernatant was measured in a Cary spectrophotometer at 620 nm. and the reading was interpolated into a standard curve of 1 to 10 /g per milliliter of Evans blue in extraction reagent. The amount of Evans blue recovered was expressed as micrograms of dye per gram of wet or dry weight of the tissue. The dry weight of the tissue was determined after dessication at room temperature.

Results. Pupil. The miotic effect observed with 0.1 per cent, 1 per cent, and 10 per cent concentration was significantly different from the controls and from one another (P < 0.01). There was no
Fig. 2. Effect of NM 0.1, 0.5, 1, and 10 per cent administered topically upon the intraocular pressure. No change was observed with the 0.1 per cent concentration. The actual pressure observed with 0.5 per cent NM was 28.7 ± 1.4 (6), with 1 per cent was 35.4 ± 1.7 (6), and with 10 per cent was 49 ± 0.8 (6). There was no effect on the contralateral side.

Fig. 3. Effect of NM administered topically upon the blood aqueous barrier. Protein concentration measured in aqueous humor 30 minutes after 0.1, 0.5, 1, and 10 per cent NM administered topically. Each point represents the mean ± S.E.M. of six animals.

contralateral effect on the pupil, with any of the concentrations. Fig. 1 shows the time course of the pupil response for each concentration. Miosis appears soon after administration of the drug. The quickest response was observed with the 10 per cent concentration, three minutes after administration.

Intraocular pressure. There was no contralateral increase in pressure at any of the concentrations of the drug. Fig. 2 shows the dose-re-
Fig. 4. Effect of NM on conjunctival vessels. Thirty minutes after topical administration of NM at 0.1, 0.5, 1, and 10 per cent Evans blue was injected intravenously. The Evans blue present in the conjunctiva from the experimental and control side was measured in micrograms of dye per gram of dry tissue and the result expressed as the ratio between experimental and control side. Each point represents the mean ± S.E.M. of six measurements.

Table I. Iris hyperemia after topical NM (Evans blue [yg] dry weight; Ratio Treated/Control)

<table>
<thead>
<tr>
<th>NM concentration (%)</th>
<th>Ratio*</th>
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<tr>
<td>0.1</td>
<td>1.90 ± 0.6 (6)</td>
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<tr>
<td>0.5</td>
<td>2.07 ± 0.4 (6)</td>
</tr>
<tr>
<td>1</td>
<td>3.0 ± 0.5 (6)</td>
</tr>
<tr>
<td>10</td>
<td>2.70 ± 0.5 (6)</td>
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*Mean ± S.E.M. (number of animals).

Hyperemia of conjunctiva and iris. Fig. 4 shows the dose-response curve for conjunctival hyperemia after NM. The values shown represent the ratio of the amount of Evans blue recovered from the treated to the control side, in micrograms of dye per gram of dry weight tissue. The values obtained for experimental and controls became significantly different at the 0.5 per cent concentration.

Table I shows ratios between Evans blue recovered from the iris of the treated and untreated eyes. The hyperemia of the treated side became significant at the 0.1 per cent concentration.

Evans blue in aqueous humor. Protein and dye concentrations in the anterior chamber were correlated. Measurements of the concentration of the dye in the anterior chamber after 1 per cent and 10 per cent NM applied topically shows two times the value for the latter, a difference similar to that found for the differences in protein concentration after these doses (Fig. 3).

Discussion. In experimental models of ocular inflammation a quantitative approach is desirable. Evans blue dye was used to quantify the hyperemia after irritation of the eye with NM. Increasing hyperemia in the conjunctiva was present after increasing doses. The method was effective in correlating dose with response. The amount of dye extracted from the iris was significantly larger in the treated side with all concentrations of the drug but an increased response after an increased dose could not be demonstrated in the iris as contrasted with the conjunctiva. Per-
haps the hyperemia of the iris is always maximal. Iris vessels are known to have zonula occludens between endothelial cells and are not fenestrated like the vessels of the ciliary body or conjunctiva. Permeability of iris vessels to horseradish peroxidase has been demonstrated only after an intense uveitis. In mild uveitis horseradish peroxidase leaks only from ciliary body vessels and across the epithelium. After topical PGE₂, the blood vessels of the iris retain their integrity and do not leak Evans blue, whereas substantial leakage of the dye into the posterior chamber and also directly into the iris stroma from ciliary processes occurs. Molecular size, of course, is critical in considerations of permeability. The molecular weight of Evans blue is 980, but evidence has accumulated that, after intravenous injection, the dye becomes firmly bound to serum albumin in a 10:1 ratio, and it escapes from circulation as dye-protein complex. This results in a molecular weight of the dye-protein complex of nearly 70,000. So the Evans blue recovered from the iris may actually represent the sum of Evans blue present in the lumen of the vessels, and that in the stroma (as in the case of fluorescein) originating from nearby ciliary processes and from aqueous humor penetrating into the anterior surface of the iris. Except for this effect on the iris, however, the results quantitatively characterize the effect of NM on intraocular pressure, blood-aqueous barrier, pupil, and conjunctival vessels.

The effect of NM requires intact innervation. Direct action of the drug on an ocular structure is probably not the cause of the irritative reaction. Pain fibers are almost certainly involved in the pathway. Although the mediator released by stimulation of these fibers is not known, the present finding of a well-characterized dose-response curve is compatible with and perhaps suggestive of the idea that the effects are the consequence of specific transmitter-receptor mechanism, perhaps for one or more substances released by these pain fibers. The data from Fig. 5, examining the dose required to produce 50 per cent of a maximal effect, possibly suggest that changes in intraocular pressure, hyperemia, and aqueous protein may require one receptor while the miotic effect on the pupil may be determined by another receptor. Further studies exploring this possibility are in progress.

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REFERENCES

Lysozyme, a basic protein with a molecular weight of 15,000, is composed of 130 amino acids. Almost 75 per cent of the total body lysozyme is concentrated within or released from polymorphonuclear leukocytes and monocytes. Previous work by Tessler and Weinberg has confirmed the presence of lysozyme in the aqueous humor and has demonstrated an increase in the level of aqueous lysozyme in certain types of ocular inflammation. The purpose of this investigation was (1) to determine the normal aqueous and vitreous lysozyme, (2) to determine if these normal levels increase after a single intravitreal injection of a foreign protein, and (3) to determine when these levels reach their maximum.

Materials and methods. Twenty-five albino rabbits weighing approximately 2 to 3 kilograms were used in this study. Four rabbits received no treatment and served to establish normal values. Seven rabbits received a single intravitreal injection of 0.1 ml. of normal saline (with a 27-gauge needle on a 1 ml. tuberculin syringe through the pars plana); anterior paracentesis relieved the subsequent rise in intraocular pressure. The 14 remaining rabbits received a single intravitreal injection of purified egg albumin (Sigma Chemical Company, grade VI) dissolved in normal saline (5 mg. per 0.1 ml.) and an anterior paracentesis. Reflux was minimal after all injections.

At 1, 3, 5, 7, 14, 21, and 28 days after injection, aqueous samples of approximately 0.2 ml. and vitreous samples of approximately 0.5 ml. were aspirated and stored in sterile tubes at 8° C. Animals were killed with sodium pentobarbital.

Aqueous and vitreous assays were performed within four days of their collection on a Coleman spectrophotometer. The lysozyme assay kit (Worthington Biochemical Corporation) containing standard lysozyme from lyophilized hen egg white and lyophilized Micrococcus lysodeikticus substrate was used in all our measurements. After reconstitution of our standard and our substrate, we adjusted our spectrophotometer to 550 nm. and prepared a standard curve of lysozyme activity. All reactions were carried out at 26° C. The change in optical density of each unknown sample was read off the standard curve to yield the concentration. All concentrations of lysozyme were determined in the aqueous and vitreous humor of rabbit eyes after intravitreal injection of a foreign protein.


We determined normal aqueous and vitreous lysozyme levels in rabbit eyes and induced experimental uveitis to record the uppermost aqueous and vitreous lysozyme levels. The normal aqueous humor of the rabbit eye contained 1.05 μg per milliliter lysozyme and the normal vitreous humor contained 0.45 μg per milliliter. After the intravitreal administration of a foreign protein, the aqueous and vitreous lysozyme levels rose within one day, reaching maximum values of 38.4 μg per milliliter and 114 μg per milliliter, respectively, at 14 days, and subsequently declining to minimal values by 28 days after injection.