tide chains, implying that different BP patients may have various sets of autoantibodies reactive with epidermal antigens. The BP serum we tested reacted with protein bands of 240 and 180 kilodaltons in rabbit corneal and human epidermal epithelium, implying that by this criteria, the BP antigen is similar in both cornea and epidermis. Similar studies with the less frequently detected autoantibodies in BP sera are being performed. Immunoblots with 24-hr-old eyebank human corneas detected similar bands at 240 and 180 kilodaltons, but several additional bands that were likely proteolytic fragments were also detected (not shown). It should also be noted that although IEM points to the HD as the site of BP antigen, the authors cannot conclude conclusively from these data that the proteins detected by immunoblotting with BP sera are in fact the same HD proteins that are identified by IF and IEM techniques.

Fujikawa et al.2 have reported a detailed analysis of BMZ components in healing rabbit corneal epithelial wounds. When the corneal epithelium was removed by a scrape wound, BP antigen was also removed. The migrating corneal epithelium lacked HD, and if the BP antigen was absent. BP antigen reappeared after migration was complete, at which time EM showed that HD had reformed. Their observation that the presence or absence of the antigen (by IF) correlates with the presence or absence of HD (by EM), provides additional correlative evidence for the authors’ finding that BP antigen is localized to the HD.

Hemidesmosomes may play an important role in the adherence of stratified squamous epithelium to the underlying stroma. Definition of HD formation, composition, and function may be important for understanding the biology of corneal epithelial adherence and migration. These findings demonstrate that the BP antigen is a component of the HD and suggest that HD formation and presence may be studied by light microscopy using IF with BP sera.

Key words: bullous pemphigoid, hemidesmosomes, immunoelectron microscopy, Western blot, cornea, wound healing

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References


Clearance and Localization of Intravitreal Liposomes in the Aphakic Vitrectomized Eye

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The authors have examined the fate of intravitreally injected liposomes in the aphakic, vitrectomized eye of the rabbit. Liposomes labelled with 125I-p-hydroxybenzimidylphosphatidylethanolamine were eliminated rapidly from the intracocular fluid. Nonetheless, a significant fraction of these liposomes were found to bind to various ocular tissues including the retina, iris, sclera, and cornea. Ultrastructural studies with gold colloid-loaded liposomes revealed that ret-
inal bound liposomes were attached to the inner limiting lamina but did not penetrate to the internal cells of the retina. Epiretinal cells bound and internalized gold colloid-loaded liposomes suggesting that these cells may be very sensitive to liposome mediated drug delivery. Invest Ophthalmol Vis Sci 28:907-911, 1987

There is growing interest in the use of chemotherapy as an adjunct to the surgical treatment of proliferative vitreoretinopathy. Clinical studies of retinal detachment in patients with proliferative vitreoretinopathy have indicated the desirability of ocular chemotherapy to control the proliferation and contraction of cells on tissue surfaces within the eye.1

Chemotherapy of proliferative vitreoretinopathy has two major limitations. It is difficult to maintain adequate intraocular levels of drug because the efflux from the vitrectomized eye is very rapid.2 It is also difficult to achieve effective therapy without toxicity to retinal cells.3

For these reasons, the authors have attempted to devise a drug delivery approach involving the use of liposomes. Liposomes might improve the efficacy of drugs for the therapy of proliferative vitreoretinopathy (PVR) by one of two mechanisms. Drug might slowly effuse from an intraocular depot of liposomes, thereby prolonging the length of exposure of epiretinal cells to the drug.4 Alternatively, the liposomes might be endocytosed and thereby might specifically deliver their contents to the epiretinal cells.5 It is important to establish which of these mechanisms is most relevant, as the choice of chemotherapeutic agent will depend on the mechanism of drug delivery.5

Materials and Methods. Phosphatidylserine was obtained from Avanti (Birmingham, AL). Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO) and recrystallized four times from ethanol. All lipids were stored at -40°C under argon prior to use. All other materials were reagent grade or better and were purchased from Sigma Chemical Co. (St. Louis, MO) and recrystallized four times from ethanol. All lipids

Liposomes were prepared from phosphatidylserine:cholesterol (1:1 molar ratio) by reverse phase evaporation.5 The mean diameter of liposomes prepared in this way is 0.5 μm.7 In order to label the lipid of liposomes, 125I](-p-hydroxybenzimidylphosphatidylethanolamine) was prepared by the method of Abra et al.8 The label was incorporated into a 1:1 mixture of phosphatidylserine:cholesterol to give 2 x 10^6 cpm/μmol lipid. The labeled mixture was then used for liposome preparation by the reverse phase evaporation method.6 Residual unbound 125I] was eliminated by gel chromatography as described above and the label content in the liposome fraction was measured with a gamma counter.

For electron microscopic studies, liposomes containing gold colloid were prepared by the method of Hong et al.9 The liposomes were composed of phosphatidylserine:cholesterol (1:1), and were made by reverse phase evaporation.6

Five New Zealand white rabbits weighing between 2.5 and 3.5 kg were used for in-vivo studies. All animal experiments conformed to the ARVO Resolution on the Use of Animals in Research. The animals were sedated with intramuscularly administered ketamine hydrochloride (20 mg/kg body weight) and xylazine hydrochloride (5 mg/kg body weight). A pars plana lensectomy and vitrectomy were performed as previously described.10 Two weeks minimum healing time was allowed before using the previously operated eyes.

Liposome clearance, binding, and distribution studies were performed with 125I] labeled liposomes. After intramuscular anesthesia with ketamine HCl (20 mg/kg body weight), topical anesthesia of the cornea was obtained with proparacaine HCl 0.5% and a preplaced mattress corneal suture of 5-0 Dacron was sewn into place. The labeled liposomes were then injected using a 30-gauge needle placed through the clear cornea into the center of the globe. The needle was then withdrawn and the preplaced suture tied. Fifty-microliter samples of intraocular fluid from the vitreous cavity were withdrawn from the eye using a sterile 30-gauge needle attached to a microliter syringe that was inserted through the previously placed mattress suture. Additional samples were taken in a similar manner. The 50-μl samples of intraocular fluid were counted in a γ counter. The ocular fluid was sampled at 1, 3, 6, 24, and 48 hr.

Animals were killed at 24 and 48 hr and the eyes enucleated. Individual portions of the globe including the cornea, iris, retina, and sclera were dissected and counted in a γ counter before and after washing three times with phosphate buffered saline. The dissection planes were made with a sharp razor blade through the clear cornea and just behind the iris to isolate cornea, iris and ciliary body, and the posterior globe. The retina was carefully peeled off the retinal pigment epithelium and cut free at the optic nerve. The dissected parts of the eyes were counted intact in a gamma counter as minimal tissue quenching occurs with 125I]. The percentage of dose in each dissected part was calculated as follows:

\[
\text{% of dose} = \frac{\text{CPM in sample} - \text{background count}}{\text{CPM in original dose} - \text{background count}} \times 100
\]

Background counts were 80 CPM.

Experimental epiretinal membranes were initiated by injecting 10,000 cultured rabbit dermal fibroblasts onto the retinas of two rabbit eyes which had previously
undergone lensectomy and vitrectomy.\textsuperscript{11} Two hours later, 0.1 ml of liposomes containing gold colloid were injected onto the retina through the clear cornea. After 24 hr, the animals were killed and the eyes enucleated and processed for electron microscopy as previously described.\textsuperscript{3}

Results. Figure 1 shows the ocular clearance of $^{125}\text{I}$ lipid labeled liposomes from the rabbit eye. Least-squares linear regression analysis of the clearance curves obtained from ocular fluid samples within the first 6 hr following intraocular injection indicates an average half life of 3.02 ± 0.4 hr. Clearance will be partially due to the sampling of the intraocular fluid, but this could only account for as much as 15% of the drug.

Table 1 shows the distribution of $^{125}\text{I}$ labeled liposomes in the various tissues of the eye 24 hr after injection. At this time, 15% of the dose remains in the intraocular fluid compartment. The tissue-associated liposomes appear to represent a component that has become firmly bound within the eye because the tissues were extensively washed before counting. Appreciable amounts of the total dose appear to have associated with the retina (8.8%), iris (5.6%), sclera (2.3%), and cornea (0.9%), the highest level being associated with the retina. The amount of $^{125}\text{I}$ in the blood was very low throughout the 48 hr of measurement and did not exceed 0.006%. Similar amounts of $^{125}\text{I}$ were associated with these ocular tissues in rabbits killed 48 hr after injection (data not shown).

Figure 2A shows the electron microscopy of eyes containing epiretinal membranes that were injected with liposomes containing colloidal gold. Cells in various layers of the epiretinal membrane contain liposomes. Many of the cells of the epiretinal membrane contain gold colloid which appears to be within the lysosomal compartment (Fig. 2B). This indicates that the cells have endocytosed the liposomes and have degraded them within the lysosomes. Liposomes also can be seen adherent to the surface of the inner limiting lamina of the retina (Fig. 2C) confirming the studies with $^{125}\text{I}$ labeled liposomes in which 8% of the dose was bound to the retina at 24 hr. Despite this high level of retinal binding, there are no liposomes or gold colloid particles within the retina. Presumably the liposomes cannot penetrate the internal limiting lamina and are not taken up by the retinal cells.

Discussion. Intraocular drug delivery using liposomes may be influenced by a variety of factors including the liposome-drug half-life within the eye, the stability of the liposome, the adherence of the liposome to the various ocular surfaces, and the passage of the liposome across tissue barriers such as the internal limiting lamina of the retina. When the authors began these studies, they were aware of two possible ways in which liposomes might improve the chemotherapy of epiretinal membranes. First, it seemed possible that liposomes might cause retention of drugs for long periods within the eye. Second, it seemed possible that liposomes might deliver the drugs specifically to the cells of the epiretinal membrane.

These studies indicate that liposomes are cleared as rapidly from a vitrectomized aphakic eye as small molecules of free drug such as fluorouracil which has a half-life in the aphakic vitrectomized eye of 3.2 hr.\textsuperscript{11} Thus, liposomes will not significantly enhance drug retention within the ocular fluid. The values that the authors have observed for the half-life of intravitreally injected liposomes in the vitrectomized, lensectomized eye are short and emphasize the problem that arises when attempting to maintain intraocular drug levels in patients with proliferative vitreoretinopathy who

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**Table 1. Distribution of $^{125}\text{I}$ labeled liposomes in the aphakic vitrectomized eye**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% dose (24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraocular fluid</td>
<td>14.9 ± 7.8</td>
</tr>
<tr>
<td>Cornea</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>Iris</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td>Retina</td>
<td>8.8 ± 1.2</td>
</tr>
<tr>
<td>Sclera</td>
<td>2.3 ± 0.8</td>
</tr>
</tbody>
</table>

* The distribution of $^{125}\text{I}$ labeled liposomes in two rabbits that were injected with 0.57 μmol lipid containing $1.4 \times 10^7$ cpm $^{125}\text{I}$ phospholipid. Ocular fluids were sampled over 24 hr and the rabbits were killed at 24 hr. The eyes were dissected and the tissues were washed three times with PBS before determination of the associated $^{125}\text{I}$ in a γ counter.
have undergone vitrectomy and lens extraction surgery. It is therefore unlikely that liposomes will enhance the chemotherapy of proliferative vitreoretinopathy by acting as a slow release system.

Studies using $^{125}$I labeled liposomes indicate that limited amounts of negatively charged liposomes are bound to surfaces within the eye. Moreover, gold containing liposomes are found in the lysosomes of the epiretinal cells. The specific intracellular delivery of drugs by liposomes is known to occur through the endocytosis of the liposomes by the cells. It therefore seems likely that liposomes may be capable of delivering drugs directly to epiretinal cells.

$^{125}$I labeled liposomes bound to the retina in appreciable amounts might raise concern over the potential retinal toxic effects of liposome bound drug. However, electron microscopic studies show that while liposomes bind extensively to the internal limiting lamina of the retina they do not appear to enter the retina. Consequently, negatively charged liposomes may enhance drug delivery to cells on the retinal surface without delivering drug to intraretinal cells, thus potentially decreasing toxicity to the retina. Electron microscopic studies also show that liposomes entered cells at different levels of the epiretinal membrane which would be important in treating multilayered epiretinal membranes.

From these studies, the authors conclude that liposomes may offer an advantageous means of delivering drug to epiretinal membranes within the eye while at the same time minimizing toxicity to underlying tissues such as the retina.

Key words: proliferative vitreoretinopathy, liposome, drug delivery, intravitreal injection, gold colloid
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