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Assessment of Ocular Irritability of Liposome Preparations

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Ocular irritability of neutral or positively charged liposomes were assessed by the Draize test, histological examination and the rabbit blinking test. The mean total score (MTS) of the Draize test showed a slight increase immediately following instillation of liposome preparations. However, it did not exceed the "practically nonirritating level", and the MTS rapidly became less than the "nonirritating level". No corneal histological alteration was observed by optical microscopy following 9 instillations of each liposome preparation. Although the neutral liposome preparation failed to increase the rabbit blinking count, the positively charged liposome preparation did so to a significant degree. The neutral liposome preparation was confirmed not to give rise to ocular irritation. However, the positively charged liposome preparation may cause pain or unpleasantness following instillation.

Keywords — liposome; egg yolk lecithin; stearylamine; ocular irritability; Draize test; histology; blinking test.

Introduction

Recently, various liposome preparations have been studied for their application in drug dosage form in ophthalmology.[1] We have formulated some liposome preparations containing anti-inflammatory steroids and examined them for their use as ophthalmic drug delivery systems.[2] The preparations were found to increase the *in vivo* corneal absorption of dexamethasone valerate.[3] The eye is not only one of the most important sense organs but is comprised of very sensitive tissues, and thus it is essential that liposomes not cause injury or irritation to ophthalmic tissues. However, few reports have been published regarding local irritability due to liposomes, and hardly any data is available on ophthalmic irritability.[4]

The Draize test, established by Draize *et al.*,[5] is generally carried out to conveniently assess ocular irritability from drugs or chemical ingredients of pharmaceutical or cosmetic preparations. However, this test is suitable for assessing severe irritation rather than weak irritation. Tanaka *et al.*[6] thus developed the rabbit blinking test for such cases, and found it capable of detecting slight irritability caused by an ophthalmic preparation below the sensitivity range of the Draize test.

In the present study, ocular irritability due to neutral or positively charged liposome preparations was evaluated by the Draize test, histological examination and the rabbit blinking test. From these results, the appropriateness of liposomal application for ophthalmology is discussed.

Materials and Methods

Materials — Egg yolk lecithin (EYL), more than 99% pure, was obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade from commercial sources.

Formulation of Liposomes — One hundred and twenty mg of EYL were dissolved in 5 ml chloroform, and 5 mg of stearylamine (SA) were added when required. The organic solvent was evaporated under vacuum to a thin lipid film which was then dried and suspended in 10 ml pH 7.4 isotonic phosphate buffer by Vortex mixing followed by ultrasonic radiation for 2.5 min using an Ohtake 5202 sonicater under nitrogen at 0 °C. After standing for a period of 1 h at room temperature, the liposome suspension was filtered through a polycarbonate membrane filter of 1 μm pore size (Nuclepore corp., Pleasanton, U.S.A.) to remove metallic foreign mat-

ters contaminated during ultrasonic radiation. The neutral liposome preparation contained 16 mM EYL and the positively charged liposome preparation, 16 mM EYL and 2 mM stearylamine. Mean liposome diameter was about 100 nm, as determined by dynamic light scattering (Submicron Sizer BI-90, Brookhaven). The liposomes were considered multilamellar vesicles.

Draize Test — Five male albino rabbits (2.0—3.0 kg) were used for one group. A 50 μl test preparation was instilled in the left eye and saline at the same time into the right eye. This was repeated every 15 min for 2 h (9 times). The appearance of the cornea, conjunctiva or iris was examined visually at 0, 1, 3, and 6 h after the last instillation. The Draize score was determined by visual assessment of changes in cornea, iris and conjunctiva.[5] The mean total score (MTS) was calculated as follows;

$$MTS = \Sigma X_1(n)/5 + \Sigma X_2(n)/5 + \Sigma X_3(n)/5$$

where $X_1(n)$, $X_2(n)$ and $X_3(n)$ are the cornea, conjunctiva and iris scores respectively and n is the number of rabbits. The degree of irritability was determined by a comparison of obtained MTS values with the data of Kay's table.[7]

Histological Examination — A 50 μl test preparation was instilled into the left eye and 50 μl saline at the same time into the right eye; this was repeated every 15 min for 2 h. Immediately following the last instillation, each rabbit was sacrificed by a rapid injection of pentobarbital sodium into a marginal ear vein. The eyes were immediately enucleated and the corneas separated, fixed in 10% formalin and embedded in paraffin for microscopical study. The sections were stained with hematoxylin-eosin (H.E.) for examination by optical microscopy.

Blinking Test — Six male albino rabbits (2.0—3.0 kg) were used for the blinking test. Each rabbit was placed in a wooden box, and 50 μl of saline was instilled in one eye and simultaneously 50 μl of test preparation into the other eye. The number of blinks of each eye per 5 min after instillation was counted. This instillation was repeated 6 times at 1 h intervals, alternating the saline or test preparation to the left or right eye. The mean of 6 experiments was taken as the estimated value.

Results and Discussion

The MTS following a single instillation of 10 μl *n*-butanol is shown in Fig. 1. The instillation caused the MTS to immediately increase to a high level ("Minimally irritating"—"Mildly irritating" level) which was maintained for at least 48 h. Walberg reported *n*-butanol caused mild ocular irritation.[8] The eye treated with *n*-butanol showed hyperemia and slight edema in the conjunctiva and some discharge, but no opacity in the cornea or no severe symptoms were evident. However, following its occurrence, ocular irritation persisted for about two days.

A comparison of MTS after 9 instillations of liposome preparations with those of the control eye treated with saline is illustrated in Fig. 2. A slight increase was immediately noted following the last instillation of each liposome preparation. However, the maximum MTS level did not exceed the "practically nonirritating" level (2.5), and immediately decreased to the "nonirritating" level (0.5). Some eyes, to which the test preparation had been applied, showed slight hyperemia in the conjunctiva, and the cornea and iris were essentially the same in appearance as the control eye. Some control eyes also

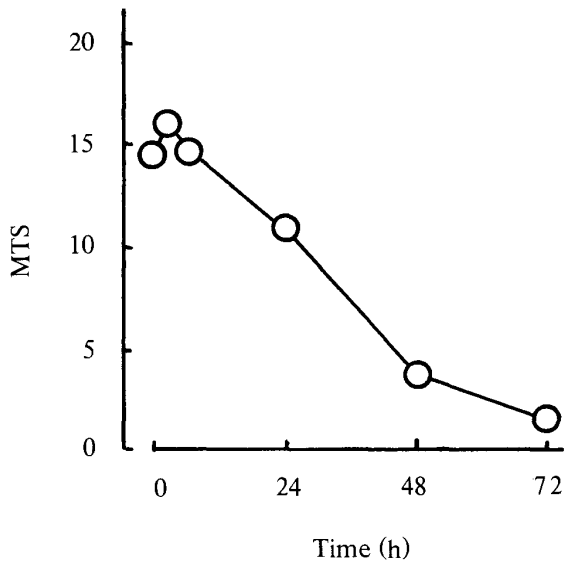

Fig. 1. Draize Score After a Single Instillation of 10 μl *n*-Butanol

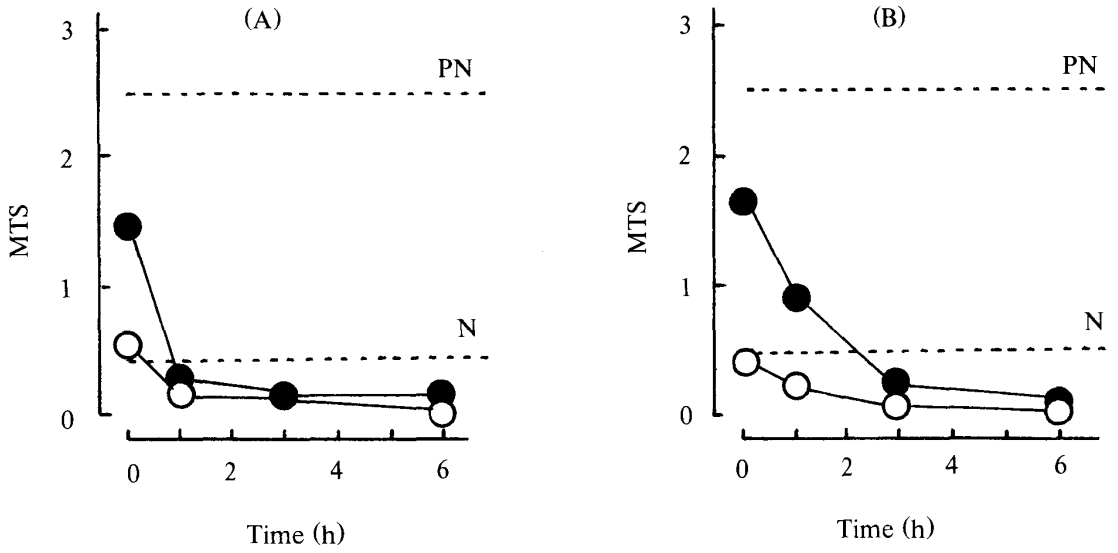

Fig. 2. Draize Score After Instillations of Liposome preparations or Saline (A), Neutral Liposomes; (B), Positively Charged Liposomes

N, upper limit of "Nonirritatating" level; PN, upper limit of "Practically nonirritating" level; ●, test eyes; ○, control eyes.

showed slight hyperemia, and the hyperemia after instillation of test preparation or saline immediately disappeared. From a practical standpoint, irritability due to each liposome preparation may be considered negligible.

The cornea is the most sensitive tissue to irritation from an ophthalmic preparation such as eye drops, and the damage incurred is difficult to assess by only external observation. However, no histological abnormality could be found in corneas treated with neutral or positively charged liposomes. With or without this treatment, cornea appearance was essentially the same and thus neither the neutral nor the positively charged liposome preparation appears to cause histological damage to the cornea.

Since the Draize test was originally designed to assess rather strong ocular irritability of materials, it would not be applicable for determining the degree of weak ocular irritability. Tanaka et al. thus established a rabbit blinking test for this purpose, and found that pH 5.9 isotonic phosphate buffer caused weak ocular irritation.[6] To detect such weak irritability from a liposome preparation, the rabbit blinking test was performed. The blinking count per 5 min following instillation of saline or the neutral liposome preparation are shown in Fig. 3. The shadowed columns show the blinking count per 5 min for each eye separately. Saline was instilled to one eye and the liposome preparation into the other at the same time. The blinking count following saline application was 4.31 ± 0.76, and that of the liposome preparation, 4.64 ± 0.63. Excluding the blinks of both eyes together, the former was 1.70 ± 0.47, and latter was 2.03 ± 0.28. These values show no statistically significant difference. The blinking count following instillation of saline or the positively charged liposome

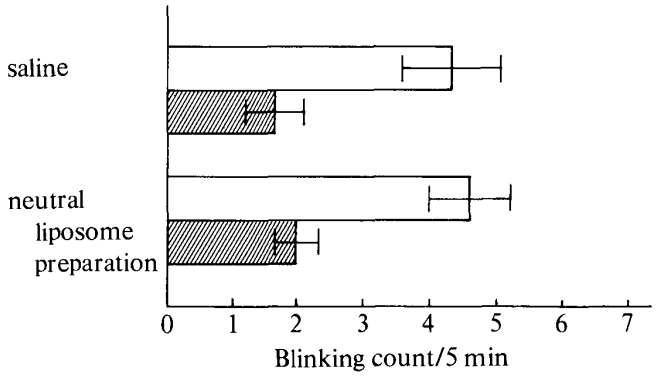

Fig. 3. Rabbit Blinking Count Following Instillation of Saline and the Neutral Liposome Preparation

Shadowed columns show the blinking count excluding the blink of both eyes together. Each bar shows mean ± S.E. of 6 animals.

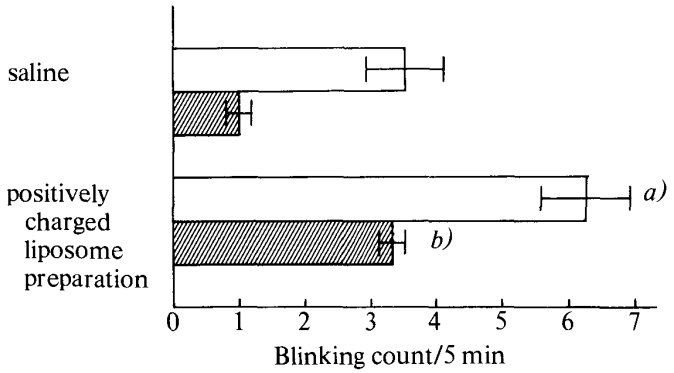

Fig. 4. Rabbit Blinking Count Following Instillation of Saline and the Positively Charged Liposome Preparation
Shadowed columns show the blinking count excluding the blink of both eyes together. Each bar shows mean ± S.E. of 6 animals.
a) $p < 0.02$, *b)* $p < 0.001$.

preparation is shown in Fig. 4. The blinking count after instillation of saline was 3.52 ± 0.27 and that of the liposome preparation was 6.27 ± 0.32, the latter being significantly higher than the former ($p < 0.02$). The blinking count excluding that of both eyes together was also significantly higher for the liposome preparation than for saline ($p < 0.001$). It is thus evident that the instillation of the positively charged liposome preparation increased the rabbit blinking count.

From the results of the Draize test and the histological examination, neither of the two liposome preparations used in this study caused local toxicity or damage to ocular tissues. Since it leads to no increase in the rabbit blinking count, the neutral liposome preparation was confirmed to be a safe ophthalmic preparation. However, the positively charged liposome preparation increased the rabbit blinking count. Consequently, it is suggested that the liposome preparation containing stearylamine may cause pain or unpleasantness following instillation to the eyes, and its continued use over long periods may lead to some problems.

Stearylamine has been generally used to prepare positively charged liposomes.[9] According to Shaffer *et al.*[10] the interaction between liposome and the corneal surface may be one of electrostatic adsorption, and positively charged liposomes are absorbed to the greatest extent. We also found the effect of a positively charged liposome preparation on corneal absorption of dexamethasone valerate to be better than that of a neutral liposome preparation.[3] However, stearylamine-containing liposomes have been reported to have cytotoxicity,[11] and we found that stearylamine caused weak ocular irritation. The local bio-compatibility of positively charged liposomes is doubtful, in spite of possibly longer retention in the eye or greater adsorption to the cornea, and it is undesirable to use stearylamine in pharmaceutical preparations. The research for a safe compound, which renders the liposomal surface positively charged, is expected.

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