A Reproducible Method for Injecting the Mouse Corneal Stroma

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An improved delivery system for injecting the mouse corneal stroma was developed. This system incorporates the following features: a repeating dispenser that eliminates inaccuracies in depressing a syringe plunger, foot activation which frees both hands for manipulating the needle and permits constant observation of the injection site, and a flexible 30-cm, 32-gauge stainless steel needle with a 30° bevel and a locking hub that resists pulsation due to back pressure while permitting freedom of motion by the operator. These injections were done while observing the cornea with a vertically mounted slit lamp, ideally suited for examining and photographing the eyes of laboratory animals. The reproducibility of the new delivery system, expressed in terms of the signal-to-noise ratio, was estimated and compared with that of a hand-held microsyringe by injecting a solution of radioactive chromium into the corneal stroma of A/J mice. The eyes were removed within 1 hr of injection, and the amount of chromium in each eye was determined in a gamma counter. The new delivery system had significantly (P < 0.05) greater reproducibility than the hand-held syringe and could be calibrated to deliver up to 0.65 µl to the mouse cornea. Invest Ophthalmol Vis Sci 32:366–370, 1991

The cornea is the focal point of inflammation in various vision-threatening diseases. Corneal inflammation can be sterile (ie, immunologically mediated), or it can be caused by pathogenic microorganisms. Permanent loss of visual acuity often results when the corneal stroma becomes vascularized, edematous, or scarred after infiltration by inflammatory cells.

To investigate the pathogenic mechanisms of corneal inflammatory diseases, many investigators have used the direct intrastromal injection of microbial and nonmicrobial antigens, activated lymphoid cells, or the molecular products of these cells.1-7 These studies have been done primarily in the rabbit cornea due to its large diameter and thickness. Unfortunately, the rabbit model has several limitations. The response of outbred animals such as rabbits is variable, and the rabbit model does not lend itself well to genetic studies. The paucity of immunologic reagents available for the rabbit renders the elucidation of the immunologic components of disease processes difficult to ascertain. In addition, rabbits are expensive to purchase and maintain, making it difficult to perform studies on large numbers of animals.

We previously described a model for the study of herpes simplex keratitis, a disease which has a significant immunologic component.8 This model uses intrastromal injections that must be accurately and reproducibly made into the mouse cornea. Intrastromal injections have been done in mice using a hand-held microsyringe.1 However, we found that the volume of fluid injected is difficult to control, and during injection, it is necessary to observe the syringe rather than the injection site. We describe a better method of injecting mice intrastromally which incorporates an improved delivery system and a slit lamp that has been specially modified for use with laboratory animals. The reproducibility of our delivery system is described, and its advantages are discussed.

Materials and Methods

Mice

These studies were done with female A/J mice (The Jackson Laboratory, Bar Harbor, ME) that were 4–12 weeks old. Before injection, the mice were anesthetized with 2 mg of ketamine hydrochloride (Vetalar; Parke-Davis, Morris Plains, NJ) and 0.04 mg of acepromazine maleate (Avco, Fort Dodge, IA) in 0.1 ml of calcium and magnesium-free Hank's balanced salt solution injected intramuscularly. The treatment of experimental animals in this study was in compliance with the ARVO Resolution on the Use of Animals in Research.
Delivery System

Our delivery system used a repeating dispenser (PB600; Hamilton, Reno, NV) that delivers 2% of the total capacity of the connected syringe with each pulse. For these studies, a 25- or 100-μl microsyringe (Gastight 1000 series; Hamilton) with a removable needle was used. The repeating dispenser was mounted on and activated by a device called an Electric Thumb (catalog No. 0142202, Hamilton). This device is controlled by a foot pedal, which frees the hands of the operator, and allows continuous observation of the injection site through the microscope. The microsyringe is fitted with a 30-cm, 32-gauge stainless steel needle with a 30° bevel and a Luer-lok (Hamilton, Reno, NV) hub. The stainless steel walls of the needle resist pulsation due to back pressure and are sufficiently flexible to permit freedom of motion by the operator. A 1.5-cm strip of larger diameter stainless steel tubing was molded to the needle approximately 2 cm from the tip to form a grip. This grip facilitated maneuvering and rotation of the needle.

Vertically Mounted Slit Lamp

All eyes were observed during the intrastromal injections through a specially modified slit-lamp biomicroscope (kindly provided by the Marco Instrument Company, Jacksonville, FL). The body of the slit lamp was oriented vertically. The animal was placed on an adjustable platform positioned directly under the objective lens of the slit lamp (Fig. 1). This apparatus provides a much better definition of the ocular tissues than a dissecting microscope and does not require holding the animal in front of the instrument as required with a standard horizontally mounted slit lamp. The light housing can be rotated to vary the angle of the incident light. The instrument is equipped with 10, 20, and 40 X objectives, which can be rotated into place, and has blue, green, and yellow filters. A semiautomatic camera equipped with a motor drive is mounted directly to the slit lamp.

Intrastromal Injection

The intrastromal injections were done essentially as described by Epstein and Stulting. One drop of proparacaine (0.5%) was applied to the eye. Fine-toothed forceps were then used to grasp the upper and lower lids simultaneously at the lateral canthus, which proposted and stabilized the globe. A 30-gauge disposable needle was used to enter the superficial stroma approximately 2 mm from the limbus. The 30-cm, 32-gauge stainless steel needle attached to the injection device was then threaded into the stroma to a point approximately 0.5 mm beyond the bevel. This long needle obviated the need for plastic or rubber tubing to join the needle to the syringe. Such tubing was subject to pulsation due to back pressure from the cornea. While resisting expansion, the narrow-gauge stainless steel needle remained flexible for easy maneuverability. After insertion of the needle, the foot pedal was depressed, activating the repeating dispenser to deliver fluid to the corneal stroma (Fig. 2).

A solution of radioactive chromium ($^{51}$Cr, 100 μCi/ml; Amersham, Arlington Heights, IL) was used...
Fig. 2. Slit lamp photomicrograph details an intrastromal injection of the cornea of an A/J mouse using the new delivery system.

to quantitate the volume of fluid injected. The eyes were enucleated, rinsed in phosphate-buffered saline, and then placed in glass gamma counting tubes containing 0.1 ml of phosphate-buffered saline. Controls were prepared by directly injecting the $^{51}$Cr solution into tubes containing 0.1 ml of phosphate-buffered saline. Counts per minute (cpm) of $^{51}$Cr were determined for each sample in a gamma counter (1272 Clini Gamma Quatro; Pharmacia LKB Nuclear, Gaithersburg, MD).

Histologic Examination of Injected Corneas

These studies were done as previously described.$^8$ To summarize briefly, the injected eyes were enucleated and immediately fixed in 10% neutral buffered formalin, and 5-μm paraffin sections were prepared. The sections were stained with hematoxylin-eosin, mounted with Permount (Fisher Scientific, Fair Lawn, NJ) and cover slipped for microscopic examination.

Statistical Analysis

The reproducibility of the delivery systems was expressed and estimated in terms of the corresponding sample signal-to-noise ratio.$^9$ The estimated signal-to-noise ratio was obtained from $x/s$ where $x$ is the sample average and $s$ is the sample standard deviation. The null hypothesis of no difference in the two ratios was tested with a (bootstrapping) resampling method.$^{10}$

Results

A total of 30 mice received intrastromal injections of $^{51}$Cr; 20 mice were injected using a hand-held syringe, and 10 were injected using the newly constructed delivery system. The reproducibility of the two delivery systems is illustrated in the Box and Whisker plot shown in Figure 3. The figure illustrates a large, asymmetric variance in the cpm obtained in eyes injected using the hand-held syringe. In this group, the median was 11,626 cpm, the lower quartile was 9114 cpm, and the upper quartile was 14,731 cpm for an interquartile range of 5617 cpm. In the group that received injections using the new delivery system, the median was 5550 cpm, the lower quartile was 4842 cpm, and the upper quartile was 5616 cpm for an interquartile range of 1074 cpm. The estimated signal-to-noise ratio for the group that received injections using the hand-held syringe was 2.18, significantly smaller ($P < 0.05$) than the corresponding ratio of 5.75 for the group receiving injections using the new delivery system. The larger ratio primarily reflects the small variance of results obtained with the new delivery system. The confidence interval for the ratio of the two variances indicated a significantly larger ($P < 0.05$) variance for the hand-held syringe and suggested a higher level of reproducibility for the new delivery system.

The average cpm of $^{51}$Cr observed in the eyes that were injected using the new delivery system was 5425, or 28% of the 19,375 cpm in 0.5 μl of the $^{51}$Cr...
solution. The values in this group ranged from a minimum of 4058 cpm (21% of the desired amount) to a maximum of 7131 cpm (37% of the desired amount). The average counts per minute of $^{51}$Cr observed in the eyes that were injected using the handheld syringe was 12,330 cpm (64% of the desired amount). However, this group had values ranging from a minimum of 58 cpm (0.3% of the desired amount) to a maximum of 27,665 cpm (143% of the desired amount).

To determine if the new delivery system was capable of delivering a larger volume of solution to the mouse cornea, a 100-$\mu$l syringe was installed in the repeating dispenser in place of the 25-$\mu$l syringe. Since the dispenser delivers 2% of the total volume of the attached syringe, it was expected that the 100-$\mu$l syringe would deliver four times the volume delivered by a 25-$\mu$l syringe. Five eyes received intrastromal injections of $^{51}$Cr using the new delivery system fitted with a 100-$\mu$l syringe. These eyes contained an average of 21,494 ± 2801 (standard error of the mean) cpm, representing 0.65 $\mu$l of $^{51}$Cr. Thus, the delivery system can be calibrated to deliver reproducibly at least 0.65 $\mu$l of solution to the mouse cornea.

In our study, the incidence of corneal perforation during intrastromal injections with the hand-held syringe was 15% compared with 2.5% when the same individual did the injections with the new delivery system. Of the successfully injected corneas (ie, those that did not perforate during injection), none had tissue damage that was discernible 48 hr after injection when examined clinically with a slit lamp or histologically using light microscopy (Fig. 4), regardless of the injection device used.

**Discussion**

The rabbit has been the preferred species for studies of corneal disease due to its relatively large eye. However, the rabbit model has several disadvantages, which have led some investigators to develop mouse models. Rabbits are outbred animals, and the response of rabbits to various corneal insults is variable. The rabbit does not lend itself well to genetic or immunologic studies, and the cost of purchasing and housing the animals can be prohibitive. These disadvantages of the rabbit model can be circumvented through the use of inbred mice. However, the small diameter and thinness of the mouse cornea has militated against the use of these animals for corneal studies. Nonetheless, with the use of the delivery system and vertically mounted slit lamp we describe, intrastromal injections can be done in the mouse cornea with accuracy and precision, and changes in the cornea resulting from these injections can be easily monitored and documented.

The pedal activation of the new delivery system enables simultaneous injection and observation of the injection site. This is extremely important when injecting into a thin and fragile mouse cornea. We observed a substantial reduction in the incidence of corneal perforation during intrastromal injections using the new delivery system compared with the hand-held syringe. A major source of injection error using the hand-held syringe stems from the difficulty of accurately depressing the syringe plunger while keeping the point of the needle inserted in the corneal stroma. The use of a Hamilton repeating dispenser to depress the plunger eliminates that source of inaccuracy.

![Fig. 4. Photomicrograph of a section of a cornea obtained 48 hr after an intrastromal injection of 0.65 $\mu$l of phosphate buffered saline using the new delivery system. This section was representative of 12 additional sections spanning the entire cornea. No structural damage to the cornea was apparent.](image-url)
Our data showed a significantly improved reproducibility of the new delivery system, compared with the hand-held syringe, for delivery of liquids into the corneal stroma. Our device incorporates a long 32-gauge stainless steel needle, which attaches directly to the syringe and obviates the need for plastic or rubber tubing to connect the needle to the syringe. The walls of the stainless steel needle are sufficiently strong to resist pulsations due to back pressure, while retaining flexibility. These features permit solutions to be injected into the corneal stroma with much greater force. Nonetheless, the average number of counts per minute of $^{51}$Cr detected in the eyes of intrastromally injected mice was 28% of that contained in 0.5 $\mu$l of the same chromium solution. This finding suggests the nonexclusive possibilities that the entire set volume of solution does not reach the cornea, or that some of the solution is lost when the needle is retracted from the cornea. Forcing a liquid through the narrow orifice of a 32-gauge needle undoubtedly creates considerable back pressure, which, when combined with the pressure in the cornea may prevent the complete injection of a small volume of fluid. We noted, however, that the movement of the syringe plunger is not measurably retarded when injecting into the cornea. Since the stainless steel needle is connected directly to the syringe via a Luer-lok hub, pulsation or leakage of fluid from the system is unlikely. It appears likely, therefore, that a portion of the injected fluid is consistently lost from the cornea on retraction of the needle. It should be noted, however, that the system can be calibrated so that a desired volume of fluid is retained in the cornea by increasing or decreasing the size of the microsyringe that is mounted on the dispenser. Our findings with the 100-$\mu$l syringe demonstrate that the mouse cornea can reproducibly retain at least 0.65 $\mu$l of solution.

In other investigations we used this delivery system to inject herpes simplex virus into the corneal stroma of mice. These injections resulted in severe stromal inflammation with no involvement of the corneal epithelium until very late in the response, when the inflammation spread into the epithelium from the stroma. With this delivery system, it will be possible to study the effects of various substances in the stroma of mouse corneas while minimizing damage to the epithelium.

**Key words:** murine, cornea, delivery system, intrastromal injection, slit lamp

**References**