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Experimental Keratitis Due to *Pseudomonas aeruginosa*: Model for Evaluation of Antimicrobial Drugs

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An improved method for experimental keratitis due to *Pseudomonas aeruginosa* is described. Essential features of the method are use of inbred guinea pigs, intracorneal injection of bacteria, subconjunctival injecion of antibiotics, "blind" evaluation of results, and statistical analysis of data. Untreated ocular infections were most severe 5 to 7 days after infection. Sterilized bacterial suspensions caused no abnormalities on day 5. Tobramycin and polymyxin B were more active than gentamicin against two strains of *Pseudomonas*. This model is suitable for many types of quantitative studies on experimental keratitis.

Pseudomonas aeruginosa has become the most common bacterial cause of severe corneal infection in man (10). The keratitis produced by *Pseudomonas* is one of the most rapidly spreading bacterial diseases of the human cornea and is one of the most destructive. Results of antibiotic therapy are not entirely satisfactory (7, 10). No controlled trials have been carried out in humans to determine optimal antibiotic therapy, and the limited studies of experimental keratitis are subject to criticism. Outbred animals have been commonly used as experimental subjects. Since *Pseudomonas* does not infect the normal cornea with an intact epithelium, the corneal epithelium must be damaged for keratitis to occur after topical application of a bacterial suspension (8). Because the severity of the corneal injury is often not uniform and because of the variability inherent in the topical application of bacterial suspensions, the severity of the keratitis in this model is unpredictable. In one recent study, untreated eyes had milder lesions than eyes treated with antibiotics (1). In another, five of 25 infected eyes treated only with saline had either no abnormalities or minimal lesions (9). Few studies have been designed so that the results could be subjected to statistical analysis with determination of confidence intervals.

We describe here an improved method for experimental *Pseudomonas* keratitis. Characteristics of this method include use of inbred animals, intracorneal inoculation of a bacterial inoculum that is standardized in terms of virulence, uniformly severe ocular lesions, subconjunctival injection of antibiotics, "blind" evaluation of results, and statistical analysis of data to determine mean effective dose (ED_{50}) with 95% confidence intervals.

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MATERIALS AND METHODS

Animals. Male strain 13 guinea pigs (Bear Creek Farms, Woodinville, Wash.) weighing 250 to 300 g were maintained in metal cages in groups of five. They were fed laboratory chow and tap water ad libitum.

Bacteria. Two strains of *P. aeruginosa* were supplied by H. B. Devlin and were identified by immunotype and a small letter as 2a and 7a (6). For animal inoculation, bacteria were grown for 24 h in brain heart infusion broth (Baltimore Biological Laboratories, Baltimore). All dilutions of bacterial suspensions were made in broth. Overnight broth cultures contained approximately 3×10^9 viable bacterial per ml as determined by colony count.

Both strains were shown to be susceptible in vitro to gentamicin, tobramycin, carbenicillin, and polymyxin B by agar diffusion tests on Mueller-Hinton agar by the Clinical Microbiology Laboratory of the University Hospital (11). Inhibition zones by the revised standard method for *Pseudomonas* 2a were carbenicillin (20 mm), gentamicin (17 mm), polymyxin B (18 mm), and tobramycin (19 mm). For *Pseudomonas* 7a, the inhibition zones were carbenicillin (22 mm), gentamicin (21 mm), polymyxin B (16 mm), and tobramycin (22 mm).

Antibiotics. Tobramycin was supplied by Eli Lilly (Indianapolis) as 40-mg/ml solutions in vials and later as a powder. Stock solutions were made up of 150 mg/ml in sterile distilled water. Gentamicin was purchased from the University Hospital Pharmacy in

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vials containing solutions of 40 mg/ml. Carbenicillin, sodium colistimethate, and polymyxin B were purchased from the Pharmacy as powders in vials. The powders were dissolved in sterile distilled water. All further dilutions of antibiotics were made in sterile saline. All antibiotic solutions were made up the day of use, and any remaining solutions were discarded. Serum tobramycin levels were determined by a radiochemical adenylation assay (12).

Toxin controls. Aliquots of overnight broth cultures of *Pseudomonas* 2a and 7a were made free of viable bacteria by heat (100 C for 3 min), passage through a 0.22-μm membrane filter (Millipore Corp.), or incubation with tobramycin (10 mg/ml) for 60 min at 37 C. Subcultures of the aliquots on agar plates had no growth.

Corneal inoculation. Guinea pigs were anesthetized by intramuscular injection of 0.8 ml of fentanyl citrate (0.4 mg/ml) and droperidol (20 mg/ml) per kg (Innovar-Vet Injection, Pitman-Moore, Washington Crossing, N. J.) and topical application of proparacaine hydrochloride (0.5%) (Ophthaine, Squibb, N. Y.). After the animals were anesthetized, the globe was held by forceps (Fig. 1). The bacterial suspension was inoculated intracorneally with a microsyringe and a 30-gauge needle in a volume of 20 μl. Antibiotics or saline were immediately injected subconjunctivally in a volume of 50 μl, except that in synergism studies carbenicillin and tobramycin were injected in a total volume of 100 μl. After the injections a boric acid ointment was applied to the cornea to prevent drying during recovery from anesthesia. Severity of the lesions was scored blindly by one of us (J.W.C.) by the criteria shown in Table 1. The criteria in the table were developed after systematically observing infected eyes for 3 weeks. Eyes with corneal perforation were given a score of 15.

Statistical analysis. Data were analyzed by the Spearman-Karber technique with the aid of a Wang computer (5).

RESULTS

Natural history of untreated infections. Groups of guinea pigs were inoculated intracorneally with various dilutions of overnight broth cultures, and eyes were examined at regular intervals for 3 weeks. These observations were the basis for the criteria shown in Table 1. Corneal edema caused by injection of fluid disappeared spontaneously in a few hours, and on the next day most eyes were normal or very nearly so. The day of injection was called day 0. Eyes infected with undiluted overnight broth cultures developed severely abnormal findings over the new few days, and corneal perforation was common by 5 days. With the more dilute suspensions such as were used for antibiotic trials, the ocular lesions on day 3 consisted of a red eye due to injection of conjunctival vessels, edema of the epithelium, and a minimal to moderately sized corneal stromal infiltrate. There was no layering of white cells in the anterior chamber (hypopyon), and there was minimal vascularization of the cornea. Findings on days 5 and 7 showed progression of the lesions characterized by increased conjunctival injection, denudation of the corneal epithelium, increased size of the stromal infiltrate, and peripheral corneal vascularization. Many coreneas had perforated by day 5, and others had perforated by day 7. The findings on day 9 were similar to those on day 7 with some animals showing improvement. Virtually all animals showed improvement by day 14 when the residual abnormalities had greatly diminished in severity. Typical findings on day 14 in eyes that had not perforated consisted of a minimal conjunctival injection, moderate corneal stromal haze, and a slight to moderate corneal vascularization.

Based on these observations, day 5 was chosen as the best day for evaluation of therapy because the ocular lesions were most severe on that day.

Reproducibility of evaluations of ocular abnormalities. Twelve guinea pigs were injected with bacterial suspensions, micropore filtrates, or saline to give a full range of ocular abnormalities. On day 5 one observer blindly evaluated the eyes twice. The second evaluation took place several hours after the first, and the observer was not aware that he was evaluating the same animals twice. The coefficient of correlation of the two evaluations was 0.93 ($P < 0.01$). Another group of 131 guinea pig eyes were evaluated blindly alive and then again from color slide photographs. At the time of evaluation from the color slides, the observer did not have access to his earlier evaluations. The coefficient of correlation of these two evaluations was 0.92 ($P < 0.01$). After this study, scores from live evaluations and color slides were pooled for analysis. However, most of the data presented in this study were based on live examinations.

Toxin controls. To determine whether it was necessary for the bacterial inoculum to contain living bacteria to cause ocular abnormalities on day 5 in this method, aliquots of a 10^{-3} dilution of an overnight broth culture of *Pseudomonas* 2a were sterilized by heat and incubated with tobramycin or micropore filtration. Two of the three corneas injected with live bacteria had perforated, and the third had a score of 5. All eyes inoculated with sterilized suspensions had scores of 0 on day 5. Similar results were obtained with sterilized 10^{-3} dilution of suspensions of *Pseudomonas* 7a.

Virulence. The virulence of overnight broth suspensions of *Pseudomonas* 2a and *Pseudo-*

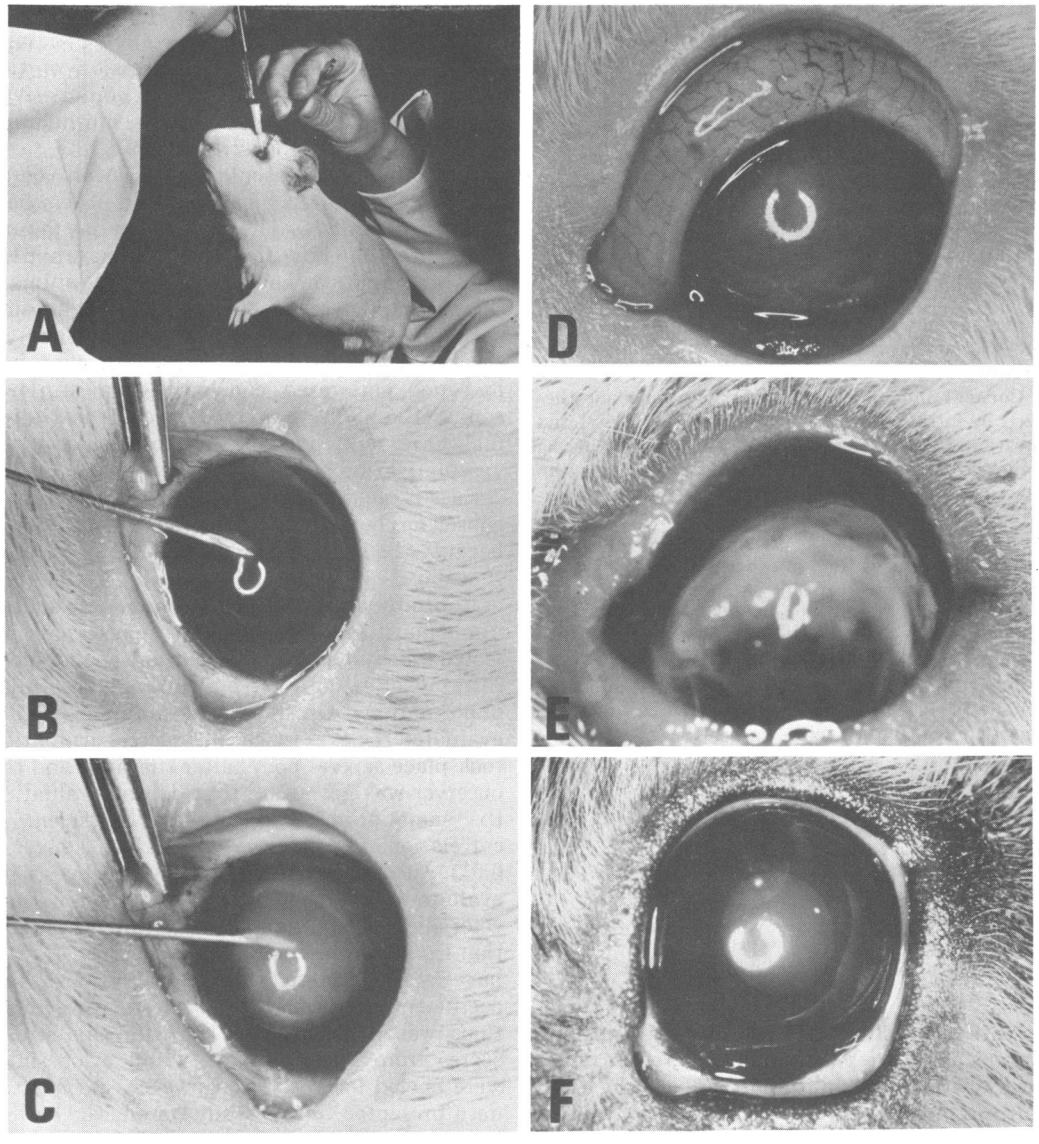

FIG. 1. *Techniques and results of ocular injections. (A) The globe is held by a forceps on the conjunctiva and solutions are injected by microsyringe and a 30-gauge needle. (B) The needle has been placed in the cornea, but no fluid has been injected. (C) Appearance of the cornea immediately after injection. The corneal opacity is due to edema and disappears spontaneously in a few hours. (D) Appearance of eye immediately after intracorneal injection and subconjunctival injection. Note the corneal opacity and the subconjunctival edema. (E) Guinea pig eye 5 days after intracorneal inoculation of Pseudomonas 2a. There was no antibiotic treatment. (F) Appearance of eye 5 days after the same intracorneal infection as in (E) except tobramycin (2 mg) was given subconjunctively.*

monas 7a was determined by inoculating guinea pig eyes with serial 10-fold dilutions of cultures. The ocular abnormalities were scored blindly on day 5 (Table 2). The inoculum for antibiotic therapy trials was arbitrarily chosen as the dilution that was 100 times the highest dilution that resulted in infections with an average score >7.5. These dilutions were 10^{-3} for *Pseudomonas* 2a and 10^{-2} for *Pseudomonas* 7a. In a preliminary trial, 5-h broth cultures of *Pseudomonas* 7a gave similar results. Overnight cultures were used as more convenient.

This standardized inoculum gave ocular infections that were uniformly severe. For the

antibiotic therapy trials, 38 eyes were infected with a 10^{-3} dilution of *Pseudomonas* 2a and treated with subconjunctival saline. The mean score of these eyes on day 5 was 13.8, and 15 of 38 eyes had perforated. No eye had a score that was less than 12. Ten eyes were infected with a 10^{-2} dilution of *Pseudomonas* 7a and treated with subconjunctival saline. On day 5, the mean score was 13.7 and three eyes had perforated. No eye had a score less than 12.

TABLE 1. *Criteria for scoring the severity of ocular lesions in experimental keratitis*[a]

Score	Reaction
Conjunctiva	
0	No reaction.
+1	Faint to moderate perilimbal injection, no edema.
+2	Marked perilimbal injection with edema.
Cornea epithelium	
0	No edema, no epithelial defect.
+1	Moderate edema, no epithelial defect.
+2	Marked edema, no epithelial defect.
+3	Marked edema, epithelial defect.
+4	Massive epithelial defect.
Stroma	
0	Clear.
+1	Stromal edema, no infiltrate.
+2	Small infiltrate, 1 to 10%.
+3	11 to 25% infiltrate.
+4	25 to 50% infiltrate.
+5	Greater than 50% infiltrate.
Vessels	
0	None.
+1	At periphery of cornea (0 to 1 mm).
+2	Vessels part way down onto cornea (2 to 3 mm).
+3	Vessels down to center of cornea (>3 mm).
Anterior chamber	
0	Clear, or cannot be evaluated.
+1	Hypopyon.

[a] Maximum score 15; corneal perforation scored as 15.

Antimicrobial therapy in vivo. To determine the relative efficacy of antibiotics in vivo, groups of guinea pigs were infected intracorneally with a 10^{-3} dilution of *Pseudomonas* 2a broth culture or with a 10^{-2} dilution of *Pseudomonas* 7a broth culture. Groups of infected animals were immediately given single subconjunctival injections of graded doses of antibiotics or saline. Results were evaluated blindly 5 days later. Results were analyzed by the Spearman-Karber technique and are shown in Table 3. For this analysis, a score >7.5 was a failure, and a score <7.5 was a cure. Tobramycin was the most active antibiotic for *Pseudomonas* 2a, and polymyxin B was the most effective for *Pseudomonas* 7a. Both were more effective for both strains than gentamicin.

In a limited study on synergism in vivo, guinea pigs were infected intracorneally with 10^{-3} dilution of broth culture of *Pseudomonas* 2a and were treated with single subconjunctival injections of a subtherapeutic dose of carbenicillin (3.25 mg) and graded doses of tobramycin. The ED_{50} of tobramycin was 0.06 mg with 95% confidence intervals of 0.02 to 0.18. These values are not significantly different from that for tobramycin alone as shown in Table 3.

TABLE 2. *Average scores of eyes 5 days after infection with serial dilutions of broth cultures*

Dilution of culture	*Pseudomonas* 2a $\overline{X}$	*Pseudomonas* 7a $\overline{X}$
Undiluted	15 (2)[a]	15 (2)[a]
10^{-1}	15 (2)	12 (1)
10^{-2}	15 (1)	13.7 (10)
10^{-3}	13.8 (38)	11 (1)
10^{-4}	14.2 (8)	8 (2)
10^{-5}	15 (1)	7 (3)
10^{-6}	3.5 (2)	3 (1)
10^{-7}	0 (2)	0 (1)

[a] Mean (number eyes).

TABLE 3. *Relative activity of antibiotics on experimental keratitis in guinea pigs with two strains of Pseudomonas aeruginosa*

Antibiotics	*Pseudomonas* 2a		*Pseudomonas* 7a	
	ED_{50} (mg)	95% Confidence intervals (mg)	ED_{50} (mg)	95% Confidence intervals (mg)
Carbenicillin	26	15-47	ND[a]	—[b]
Colistimethate	1.7	1.2-2.4	ND	—
Gentamicin	0.8	0.5-1.4	1.8	1.1-2.8
Polymyxin B	0.3	0.2-0.5	0.23	0.13-0.41
Tobramycin	0.14	0.09-0.24	0.46	0.24-0.89

[a] ND, Not done.
[b] —, Not applicable.

The data were examined to determine whether antibiotic therapy of one eye might influence the course of untreated infection in the other eye. A total of 14 guinea pigs was infected bilaterally, treated with saline in one eye and more than 0.5 mg of tobramycin, gentamicin, or polymyxin B or more than 10 mg of carbenicillin in the other eye. On day 5, the mean score of this group was 14 and eight eyes had perforated. Only one eye had a score less than 12 and it was 9.

Subconjunctival injection of antibiotic did achieve significant blood levels. A dose of 2 mg of tobramycin in one eye gave a blood level of 12 μg/ml at 2 h and 6.5 μg/ml at 5 h. A dose of 2 mg of tobramycin in both eyes gave a level of 19 μg/ml at 1.5 h and 14 μg/ml at 4.5 h.

It was concluded that antibiotic therapy in one eye was unlikely to influence the course of infection in the other eye, except when the first eye is treated with a dose several times the ED_{50}. Therefore our studies were planned so that, whenever one eye was given a large dose of antibiotic, the second eye was used as a saline or toxin control. This practice does not exclude all possibility of the therapy of one eye influencing the infection in the other eye, but it does minimize such possible influence while allowing the maximum use of animals.

DISCUSSION

Our primary goal was to develop an improved method for experimental *Pseudomonas* keratitis, and we believe this goal has been largely achieved. An inbred animal, the strain 13 guinea pig, was chosen to reduce the variability that is inherent in studies on an outbred animal such as the rabbit. Intracorneal inoculation of bacteria assured greater precision in the size of the bacterial challenge. The bacterial inoculum for antibiotic trials was defined in terms of virulence so that results from several strains may be more nearly comparable. There is evidence both in vitro and in vivo that the size of the bacterial inoculum influences the apparent activity of antibiotics (2.4). Use of a heavy bacterial inoculum might mask an important therapeutic effect. An inoculum that is too light may not produce consistently severe lesions. Antibiotics were given subconjunctivally to reduce the variability that is inherent in the topical application of drugs. The results were scored blindly to reduce observer bias. Calculation of the activity of antibiotics in terms of the ED_{50} and the 95% confidence intervals allows a more precise determination of the relative efficacy of the drugs.

In the results available for two strains, tobramycin and polymyxin B were more active in vivo than gentamicin. In a limited study on one strain, there was no evidence in vivo for synergism between tobramycin and carbenicillin.

Colistimethate was relatively ineffective in the treatment of experimental keratitis by one strain of *Pseudomonas*. In an earlier study in mice, colistimethate was significantly less effective than tobramycin or gentamicin (2). In a more recent study (3), colistimethate was distinctly inferior in the mouse protection tests with four strains of *P. aeruginosa* when compared to polymyxin B sulfate. Colistimethate appears to be significantly less effective in vivo than polymyxin B sulfate in the treatment of experimental *Pseudomonas* infections.

In beginning work on experimental keratitis, we deliberately chose to first evaluate the effects of chemotherapy given immediately after infection. Immediate chemotherapy avoids a second dose of general anesthetic and is analogous to the usual method for the mouse protection test (2). In the future, we plan to evaluate the effects of delayed chemotherapy.

The finding that sterilized broth suspensions did not cause ocular abnormalities on day 5 tends to exclude toxins contained in the original inoculum as a cause of the lesions. The fact that antibiotic therapy prevents ocular abnormalities also supports this conclusion.

Although this experimental model was developed to study experimental *Pseudomonas* infections, it should be suitable for qantitative studies on a variety of problems such as infections by other bacteria, viral infections, pathogenesis of infections, and role of host defense mechanisms.

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LITERATURE CITED

1. Bohigian, G., M. Oleumoto, and M. Valenton, 1971. Experimental *Pseudomonas* keratitis. Arch. Ophthalmol. **86**:432-437.
2. Davis, S. D. 1974. Dissociation between results of in vitro and in vivo antibiotic susceptibility tests for some strains of *Pseudomonas* aeruginosa. Antimicrob. Agents Chemother. **5**:281-288.
3. Davis, S. D. 1975. Activity of gentamicin, tobramycin, polymyxin B, and colistimethate in mouse protection tests with *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **8**:50-53.
4. Ericsson, H. M., and J. C. Sherris. 1971. Antibiotic sensitivity testing. Report of an international collaborative study. Acta Pathol. Microbiol. Scand. **217**(Suppl.):1-90.
5. Finney, D. J. 1964. Statistical analysis in biological assay,

2nd ed., p. 524. Hafner Publishing Co., New York.
6. Fisher, M. W., H. B. Devlin, and F. J. Gnabasik. 1969. New immunotype schema for *Pseudomonas* aeruginosa based on protective antigens. J. Bacteriol. **98**:835–836.
7. Hessburg, P. D. 1969. Management of *Pseudomonas* keratitis. Survey Ophthalmol. **14**:43–54.
8. Hessburg, P. D., J. P. Truant, and W. P. Penn. 1965. *Pseudomonas* corneal ulcers in animals and man, p. 752–758. Antimicrob. Agents Chemother. 1964.
9. Hessburg, P. D., J. P. Truant, and W. P. Penn. 1966. Treatment of *Pseudomonas* keratitis in rabbits. Am. J. Ophthalmol. **61**:49–54.
10. Laibson, P. R. 1972. Annual review. Cornea and sclera. Arch. Ophthalmol. **88**:553–574.
11. Reller, L. B., F. D. Schoenknecht, M. A. Kenny, and J. C. Sherris. 1974. Antibiotic susceptibility testing of *Pseudomonas aeruginosa*: selection of a control strain and criteria for magnesium and calcium content in media. J. Infect. Dis. **130**:454–463.
12. Smith, D. H., B. Van Otto, and A. L. Smith. 1972. A rapid chemical assay for gentamicin. N. Engl. J. Med. **286**:583–586.