It is of interest that AMP stains are more positive in toxin-treated corneas than in controls. Since the colloidal iron stain demonstrates nuclear fragments as well as proteoglycans, disintegrating nuclei may give a false impression of enhanced proteoglycan content.

The possibility of neutralizing exotoxin A by horse-serum antitoxin as a therapeutic approach remains to be studied.

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Key words: Antitoxin, collagen, cornea, corneal ulcer, endotoxin, exotoxin, protease, proteoglycan, *Pseudomonas aeruginosa.*

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Oral vaccination and multivalent vaccine against *Pseudomonas aeruginosa* keratitis.

JOHN R. GERKE AND JIM S. NELSON.

Active immunization against Pseudomonas aeruginosa *keratitis and systemic disease in mice was studied. In the first series of experiments, monovalent vaccine, administered orally or intraperitoneally, protected against subsequent corneal and intraperitoneal challenge with the homologous strain of* P. aeruginosa; *however, oral administration of vaccine elicited less protection than intraperitoneal administration. After both routes, protection was observed at 11 and 32 days post-vaccination, but it was greater at 11 days. In the second series of experiments, multivalent vaccine administered intraperitoneally protected against corneal challenge with 56 to 78 percent of 18 strains.*

Pseudomonas aeruginosa keratitis following trauma to the cornea may result in blindness. In addition, pseudomonal septicemia and pneumonia often lead to death. Improvements in antimicrobial therapy have reduced these threats; however, additional means are needed.

The potential of active and passive immunization against *P. aeruginosa* keratitis was first shown in 1927.[1] Active and passive immunotherapy based on a multivalent vaccine have shown promise in combating disease caused by a diversity of antigenic types of *P. aeruginosa.*[2] In some types of disease, the parenteral vaccine causes severe reactions.[2] Oral vaccine offers reduced risk of vaccine reactions.

This study was designed to determine the answer, in part, to two questions. Does immunization with a multivalent vaccine protect against experimental keratitis caused by diverse strains of *P. aeruginosa*? Does oral immunization protect against *Pseudomonas* keratitis?

Materials and methods.

P. aeruginosa cultures. The strains (Table III) were selected for their ability to damage mouse corneas and to represent the seven Fisher, Devlin, and Gnabasik immunotypes. George Cole of Parke, Davis and Co. (Detroit, Mich.) kindly provided the immunotyping information. Four of the strains have been reported on previously: strains 119 and 120 were identified[3] as PA7 and PA103, and strains 186 and 187 are respectively the type 6 and type 7 components of the heptavalent vaccine Pseudogen (Parke, Davis and Co.). All strains were maintained in 10 percent glycerol–

nutrient broth at -40° C. To grow inoculum for infecting corneas and grow cells for preparing vaccines, we used trypticase soy agar (Baltimore Biological Labs., Cockeysville, Md.) and for corneal culture, trypticase-yeast extract (TYE) agar.[4] All cultures were incubated at 37° C.

Mice. Female mice, 6 to 18 weeks old, from our colony were used. The strain was derived from a cross of albino Swiss to C57BL/6 mice. Because of the possibility of immunizing with indigenous *P. aeruginosa* in the drinking water, the water was acidified, 2×10^{-3} N HCl. The contents of used bottles were cultured, and only mice that had a history of *P. aeruginosa*–free water bottles were used. Mouse genetic diversity and age range were equalized within each treatment group by distributing litter mates equally among all groups.

Vaccine production. Bottles containing 50 ml. of agar were inoculated and incubated aerobically for 20 hours. The resulting growth was suspended in saline, and cell number estimated from the absorbance at 540 nm. The suspension was diluted to an absorbance of 2.0 and adjusted to a phenol concentration of 1 percent. After 1 to 1½ hours at 37° C., the suspension was centrifuged, and the residue was suspended in saline to the desired cell density.

For the first series of experiments, a monovalent vaccine (strain 119) was used and for the second series, a multivalent vaccine was used. The seven strains, one for each immunotype, used in the multivalent vaccine are shown in Table III.

Replication of experiments. The first experiment was repeated four times, each 2 to 4 months apart. The second experiment was run once.

Intraperitoneal vaccination. Each mouse received a 0.2 ml. dose of vaccine daily Monday through Friday for 3 weeks. The initial dose, 6×10^7 cells, was doubled each Monday, Wednesday, and Friday, achieving a final dose of 1.5×10^{10}. On Tuesdays and Thursdays the doses of the previous day were repeated. The mice were lethargic for several hours after vaccination with the larger doses, and in the third of the four repeats of the first experiment, 14 of 66 mice died following the largest or second largest dose. This schedule was chosen because it evoked more consistent protection against corneal challenge than schedules using less vaccine.

Oral vaccination. The schedule was the same as for the intraperitoneal route except that the dose volume was 10-fold lower, 0.02 ml. per mouse, and the cell densities were correspondingly 10-fold higher. When the mice were held inverted, their mouths opened partially. When the drop of vaccine touched their lips, they licked it off.

Intraperitoneal challenge. Eleven and 32 days after the last vaccination, 15 mice of each treatment group of the first experiment were challenged with intraperitoneal injections of cells of strain 119 grown on an agar slant for 16 hours. In the first repeat, the initial challenge was at 15 days instead of 11 days. Five doses, three mice each (0.2 ml./mouse) were used for each challenge. Doses of 4.0×10^6, 1.2×10^7, 4.0×10^7, 1.2×10^8, and 4.0×10^8 cells were used for nonvaccinated mice. The dose range for intraperitoneally vaccinated mice was 1.2×10^8 to 1.2×10^{10} and for the orally vaccinated, 1.2×10^7 to 1.2×10^9.

The doses and the cages of mice were coded so that the person injecting and observing the resulting mortalities did not know the dose or treatment. The mice were observed for mortalities daily for 1 week; however, all mortalities occurred before the third day.

LD_{50}'s were calculated for each treatment group, and protective indexes were determined by dividing the LD_{50} for a vaccinated group by the LD_{50} for the nonvaccinated.

Corneal challenge. For the first experiment, separate groups of mice were challenged with strain 119 at 11 and 32 days after the last vaccination. In the first repeat of the first experiment, the initial challenge was at 15 days instead of 11 days. For the second experiment, all challenges were at 11 days after the last vaccination. Both corneas of the mice of each treatment group were cultured, by loop, on TYE agar within the week prior to challenge. Only mice with negative cultures (greater than 90 percent of the mice) were used for corneal challenge. From each treatment of each repeat of the first experiment, we selected a group of 5 to 7 mice. For the second experiment, each group contained vaccinated and nonvaccinated mice, 3 to 5 of each, and the vaccinated mice of each group were from the same litters as the nonvaccinated.

The mice of a group were caged together and the treatment coded. All mice in the same cage were infected with the same strain. Both corneas were incised as described previously,[4] and saline or culture (2×10^9 cells/ml.) was dropped onto each. Approximately equal numbers of right and left eyes within each treatment were infected. The person infecting and observing did not know if the mouse had been vaccinated or which eye was infected. Mice were anesthesized intraperitoneally with sodium pentobarbital, 0.07 mg./gm., during infecting and subsequent observations. Corneas were examined by corneal culture and by visual observation with a 45× stereoscopic microscope, immediately prior to infection and at 1 day, 3 days, and weekly for the first 2 months after infection. Also, many of the corneas were observed at 3 and 4 months. The observer sketched

Table I. Corneal damage in mice treated with monovalent vaccine

	Challenged 11 days postvaccination				*Challenged 32 days postvaccination*			
	*Severity, at observation period of largest area of opacity**		*Residual opacity at 8 weeks after challenge*		*Severity, at observation period of largest area of opacity**		*Residual opacity at 8 weeks after challenge*	
Vaccine route	*Fraction with opacity†*	*Av. area of opacity (percent)*	*Fraction with opacity†*	*Av. area of opacity (percent)*	*Fraction with opacity†*	*Av. area of opacity (percent)*	*Fraction with opacity†*	*Av. area of opacity (percent)*
None	22/22	98	22/22	79	24/24	99	24/24	95
Intraperitoneal	12/23	24	2/23	1‡	20/23	32	13/23	12‡
Oral	21/22	65	14/22	19‡	24/24	82	21/24	36‡

*The first observation of the largest area of opacity of individual corneas was usually (35 of 46) the first day. With others it occurred within the first 2 weeks.

†Number of corneas with opacity to number inoculated.

‡The area of opacity for each cornea at 8 weeks (not shown) was examined by Mann-Whitney U tests. The intraperitoneal and oral routes were better than no vaccine ($p < 0.05$). Protection persisted at 32 days but was smaller than at 11 days. The decrease was significant ($p < 0.05$) for both vaccine routes.

each cornea and estimated area of dense and nondense opacity. If the pupillary margin or iris detail was visible through the opacity, it was nondense. A second person confirmed observations.

Presence and area of opacity were the least subjective of the observations, and because even a diffuse opacity degrades vision, we chose presence and area of opacity (dense plus nondense) to be the principal measurements for this report. Data for area of dense opacity alone and for corneal culture recovery of *P. aeruginosa* were analyzed, and the results were parallel to those shown in Tables I and III.

We decided to use the 8 week observations as terminal, because subsequent changes in area of opacity were insignificant. Specifically, in the first experiment, 90 percent of the mice showing opacity at 8 weeks were observed again at 16 weeks and the average area of opacity had decreased only 3 percent.

Results.

Corneal challenge of mice treated with monovalent vaccine administered by the intraperitoneal or oral route. The incised but noninoculated contralateral eyes served as controls for possible damage due to indigenous opportunists entering the incised cornea or damage caused by the incision alone. Of the 138 incised but noninoculated control eyes, only three showed damage at any observation period, and these were presumed to be contaminated from the infected eye, because the damage was typical for *P. aeruginosa* infection and *P. aeruginosa* was recovered in corneal culture. For the remaining uninoculated control eyes, damage was no longer detectable the day following incision, thus demonstrating that damage in the infected eyes resulted from the infection and not from incision alone.

The course of damage of the infected corneas of nonvaccinated mice (Table I) was quite uniform. Severity of inflammation, as indicated by the relative areas of dense and nondense opacity (not shown), reached a maximum at about 7 days. When 100 percent of a cornea showed dense opacity in the first week (44 of 46), it usually persisted through the eighth week; however, some (5 of 44) became largely nondense within 8 weeks. Corneal protrusions, ulcers extending into the stroma, and descemetoceles were common, but none of the corneas ruptured. Corneal cultures were all negative for *P. aeruginosa* beyond the seventh day of observation.

The corneal observations of inoculated eyes of the nonvaccinated and vaccinated mice are summarized in Table I. The initial severity and course of the keratitis is represented by data from the observation time first showing the largest area of opacity. Mice vaccinated by either route had less severe initial damage than did the nonvaccinated. Vaccination by either route also minimized residual opacity.

Intraperitoneal challenge of mice treated with monovalent vaccine administered by the intraperitoneal or oral route. These tests were included to illustrate by a conventional means, intraperitoneal challenge with the vaccine strain, the degree of immunity evoked by the vaccination procedures. The average protective indexes are shown in Table II. Survivors challenged with *P. aeruginosa* immunotypes differing from the vaccine strain were not protected.

Corneal challenge of mice treated with polyvalent vaccine administered by the intraperitoneal route. For each of 18 strains, a group of four vaccinated and four to five nonvaccinated mice were infected. The corneal damage in the nonvaccinated was similar to that described for the first experiment, with the exception that some of the corneas infected with strains 31, 102, 103, 108, 112, 116, 120, and 187 ruptured.

The course of the keratitis in the vaccinated and nonvaccinated was similar to that shown in Table I. Therefore, only the most critical

observation, average area of opacity at eight weeks after challenge, is shown in Table III. Based on the two criteria, statistically significant protection and possible protection, prophylactic multivalent vaccination protected against 56 to 78 percent of the 18 strains (Table III). All four strains for which there was no evidence of protection were nonvaccine strains (31, 108, 111, and 120) indicating that the vaccine was deficient in one or more elements.

The possibility that the vaccine did not protect against strains that ruptured corneas was examined (Table IV). No relationship was shown between vaccine protection and the ability of the infecting strain to cause ulcers.

Discussion. Prophylactic vaccination could be valuable for those subject to a high incidence of *Pseudomonas* keratitis, and hyperimmune serum from the vaccinated could aid in treating the nonvaccinated.

Immunoprotection by oral vaccination against *P. aeruginosa* has not been reported previously. Although the protection we demonstrated by the oral route was less than that achieved by the intraperitoneal route, it demonstrated the feasibility of using the oral route as a possible means for limiting vaccine reactions.

The feasibility of using polyvalent vaccine prophylaxis to protect against corneal damage by diverse antigenic types of *P. aeruginosa* is pointed out by the results of the second experiment. The multivalent vaccine results could have been more pertinent if the mice had been vaccinated by the oral route; however, we chose to use the intraperitoneal route, because it produced more decisive results and required fewer mice. Parallel efficacy of the intraperitoneal and oral routes demonstrated in the first experiment causes us to believe that the multivalent vaccine results would apply to a future, superior oral vaccine and regimen.

Further experimentation with orally administered polyvalent vaccine is needed: active protection, passive protection, and improvement of composition. For improving composition, our preliminary experiments suggest using formalin-killed or freeze-killed cells. Also, specific extracellular and intracellular products of *P. aeruginosa* have been implicated in disease.[5-10] Improvement could result from adding one or more of such products to the vaccine.

Demonstration that oral vaccination protected against infection is significant to experiments on *P. aeruginosa* in the mouse and possibly other animals. *P. aeruginosa* grows readily in drinking water containers, and failure to control such growth could result in oral vaccination and cause biased and variable experimental results.

From the Oregon Zoological Research Center, Portland Zoological Gardens, Portland. Supported

Table II. Intraperitoneal protective indexes in mice treated with monovalent vaccine

Vaccine route	*Challenge time postvaccination (days)*	*Average protective index**
None	11	1†
	32	1†
Intraperitoneal	11	162‡
	32	58‡
Oral	11	10‡
	32	6‡

*Ratio of the LD_{50} of the vaccinated group to that of the nonvaccinated group.

†The LD_{50}'s for the nonvaccinated at 11 days was 2.5 and at 32 days, 2.7×10^7 organisms.

‡Mann-Whitney U tests of the LD_{50}'s (used to calculate the protective indexes) showed the protective effect of both routes to be significant ($p < 0.05$) relative to the nonvaccinated group. The effect for the intraperitoneal route was greater ($p < 0.05$) than for the oral. Protection decreased somewhat between 11 and 32 days, but the decrease was statistically reliable ($p < 0.05$) only for those vaccinated by the intraperitoneal route.

Table III. Protection with heptavalent vaccine, corneal damage in mice treated with multivalent vaccine

Infecting strain	*Type*	*Average area of opacity (percent) at 8 weeks after challenge*	*Evidence for protection*
119*	1	20 (88)†	Significant‡
111	1	97 (86)	None
143	1	38 (100)	Significant‡
112*	2	3 (80)	Significant‡
31	2	78 (100)	None
120	2	100 (100)	None
113*	3	18 (100)	Significant‡
103	3, 7‖	38 (100)	Possible§
114*	4	16 (88)	Significant‡
102	4	13 (100)	Significant‡
138	4	43 (100)	Possible§
189*	5	55 (100)	Possible§
95	5	35 (78)	Significant‡
109	5	55 (100)	Possible§
186*	6	0 (85)	Significant‡
116	6	25 (100)	Possible§
108	6	100 (100)	None
187*	7	3 (100)	Significant‡

*One of the strains of the vaccine.

†Results for the nonvaccinated are in parentheses.

‡Mann-Whitney U test ($p < 0.05$).

§Not statistically significant, but would be significant if the same average were obtained with one to three more mice per group.

‖Strain 103 reacted with typing sera for type 3 and type 7.

Table IV. Multivalent vaccine protection against strains that ruptured corneas

Evidence of protection	
Significant	*None*
Infecting strains 102, 103, 112, 116, 187	Infecting strains 31, 108, 120

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Influence of anesthetics, ethyl alcohol, and Freon on dark adaptation of monkey cone ERG.

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*Cone dark adaptation curves were measured in a rhesus monkey using the electroretinogram (ERG) response to a 40 Hz flickering stimulus. The influence of anesthetics on the time course of dark adaptation was studied. All volatile anesthetics tested (methoxyflurane, halothane, enflurane, ether, chloroform) retarded dark adaptation but to different degrees; urethane, ethyl alcohol, and Freon 11 also retarded dark adaptation. No effect was found for barbiturates and ketamine. It seems unlikely that metabolites play a role in the observed phenomena. A literature survey reveals that several studies on dark adaptation or visual pigment regeneration might have suffered from influences of the anesthetic used. The cause of the phenomenon might lie either in anesthetics-induced membrane changes or in hindrance of the isomeration of 11-*trans *retinal to 11-*cis *retinal.*

Recently we[1] described that the general anesthetic halothane retarded cone dark adaptation (DA) in human subjects, macaque monkeys, and birds. Although we used a measuring method based on the electroretinogram (ERG), it was speculated that the origin of the retardation would lie in a retarded regeneration of the visual pigment. This has now been confirmed by retinal densitometry.[2] The present study was undertaken to test a series of commonly used general anesthetics with regard to their influence on cone DA.

Methods. Stimulus and recording methods were identical to the ones described by Norren and Padmos.[1] The corneal ERG to a yellow (λ = 577 nm.), 45 deg. stimulus was kept, by means of a feedback on the light intensity, at a constant level (1 μV). A continuous record of the light intensity provided a measure of the retina's sensitivity. The bleaching light consisted of a 70 deg., 6.2 log td. yellow light (Schott OG 550) presented during 5 to 10 min. Dark adaptation curves, representing the recovery of the retina's sensitivity after the bleaching light was extinguished, resembled exponential functions, and therefore, the rate of DA could be characterized by a single datum: the time constant (1/e value).

The subjects were rhesus monkeys of between 2.5 and 6 kilograms. The monkeys received phencyclidine, 0.4 mg. per kilogram (Table I) intramuscularly (I.M.), or 10 mg./kg. of ketamine I.M. as sedation. Intravenous (I.V.) injection of 1 to 2 mg./kg. of methohexital, a short-acting barbiturate, allowed further preparation of the animal. After intubation the animal received an initial I.V. dose of 30 μg/kg. of the muscle relaxant pancuronium bromide, followed by a continuous injection of 30 μg/kg. per hour of the same agent. Artificial respiration was then started. The gas mixture consisted of 70 per cent N_2O and 30 per cent O_2. In the rest of the report this is referred to as "the basic anesthesia." The N_2O was replaced by oxygen only on rare occasions in order to avoid stress for the animal. The gas flow was regulated such that expiratory CO_2 was kept between 4.0 and 5.5 per cent. At the beginning of the experiment and about 4 hours later 0.1 mg./kg. of atropine sulfate was given to suppress salivation.