consistently seen was along the inner retinal surface and along cell membranes of the inner retina (Fig. 4). This localization of enzyme activity had also been noticed in earlier experiments with cyclic AMP as substrate.1

Discussion. The previous difficulties in demonstrating cyclic GMP phosphodiesterase activity histochemically in retina appear to have been due to excessive fixation of the tissue. Glutaraldehyde fixation for more than brief periods evidently denatures or alters the enzyme in such a way that it will not react with cyclic GMP. The hydrolysis of cyclic AMP is not as sensitive to fixation, but it too is reduced by prolonged exposure to glutaraldehyde.1 I have recently used the 3 min fixation time for both cyclic AMP and cyclic GMP and find slightly more reaction product with the former when the conditions of incubation are otherwise similar. The distribution of reaction product on the outer segments is identical with the two substrates, a finding which suggests that one and the same enzyme may be capable of hydrolyzing both cyclic nucleotides.

No precipitate was seen consistently on photoreceptor inner segments, nuclei, or synaptic areas. On the vitreal side of the retina the restriction of precipitate to areas near the retinal surface may reflect limited diffusion of reactants, as discussed previously.1 The fact that the reaction product was found beneath the basement membrane of the Müller cells rather than on its surface suggests that the precipitate reflects true enzyme activity and not an artifactual deposit.

From the Departments of Ophthalmology, The Children's Hospital Medical Center and Harvard Medical School, Boston, Mass. This work was supported by U. S. Public Health Service grant 5R01 EY01451 from the National Eye Institute, Bethesda, Md. Submitted for publication Feb. 3, 1978. Reprint requests: Dr. Richard M. Robb, Department of Ophthalmology, The Children's Hospital Medical Center, 300 Longwood Ave., Boston, Mass. 02115.

Key words: phosphodiesterase, cyclic GMP, histochemistry, retina, photoreceptor cells

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The role of hemolysin in corneal infections with Pseudomonas aeruginosa. Mary K. Johnson and James H. Allen.

Cultures of Pseudomonas aeruginosa considered to be of proven virulence were found to have higher titers of extracellular hemolysin than cultures of lesser virulence. Intracorneal injection of purified hemolysin produced extensive corneal opacification with extensive leukocytic infiltration of the tissue. It is suggested that the hemolysin plays a role in the pathogenesis of P. aeruginosa infections by effecting lysis of host cells and/or subcellular organelles, leading to the release of enzymes destructive to corneal tissue.

The role of various factors elaborated by Pseudomonas aeruginosa in the destruction of ocular tissue has been studied by several investigators. Fisher and Allen1,2 produced ulceration of the cornea with cell-free extracts and with partially purified protease preparations from this organism. More recently, the role of proteolytic enzymes in Pseudomonas ulcers has been studied by other investigators.3,4 The production of damage to corneal ground substance by enzymes from Pseudomonas was reported by Brown et al.5 and by Gray and Kreger.6 The possible role of host enzymes in tissue degradation was discussed by the latter authors and further explored by Kessler et al.,7 who concluded that corneal destruction by P. aeruginosa depends not only on the protease, which rapidly destroys the cornea, but also on host-derived enzymes which are capable of degrading both collagen and proteoglycans.

In a preliminary survey in which we compared various characteristics of strains of P. aeruginosa of proven virulence with those of strains of undetermined virulence, we noted that the former appeared to be more hemolytic on human blood

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agiar. The present study explores the possible relationship of hemolysin to the host-derived enzyme activity discussed above.

**Materials and methods.** Strains of *P. aeruginosa* were obtained from Dr. Bruce Golden of the University of Iowa, Iowa City, Dr. G. Richard O'Connor of the University of California, San Francisco, and from the Department of Ophthalmology in our institution. Those isolated from corneal ulcers or cases of postoperative endophthalmitis were designated as strains of proven virulence. Strains isolated from normal eyes or those with mild conjunctivitis were considered as strains of undetermined virulence. Identification of isolates as *P. aeruginosa* was confirmed by oxidase test, pigment production on Mueller-Hinton agar, and growth at 41° C. Cultures were maintained on Pai's medium and transferred every 3 months.

The hemolysin-producing capacity of each strain was assayed by the method of Liu as modified by Berk for the extracellular heat-stable hemolysin of *P. aeruginosa*. This procedure involves cultivation of the organism on a cellophane membrane on tryptone-glucose agar for 3 days. The organisms were then washed off the cellophane with buffer, followed by centrifugation of the suspension to remove the cells and determinations of the titer of the supernatant fluid by a twofold dilution method using sheep erythrocytes.

Purified hemolysin was kindly donated by Dr. A. D. Larson. The method of purification (involving adsorption of the lysin from culture fluid onto charcoal, elution with ethanol, and precipitation with 5M NaCl) has been previously described, and the preparation is free of proteolytic activity. The hemolysin is stable to autoclaving and is similar, although not identical, to hemolytic glycolipid studied by Sierra. The purified lysin preparation had a titer of 800, which indicates the highest dilution at which 1 ml will completely hemolyze 1 ml of a 1% suspension of bovine erythrocytes in 2 hr at 37° C.

The effect of the purified lysin on the rabbit cornea was tested by intracorneal injection of 0.05 ml of sterile material dissolved in isotonic saline, with a 30-gauge needle. At least two rabbits were used for each dilution tested. In each case one eye served as control and was injected with an equivalent volume of sterile physiological saline. Control eyes showed only a slight transient corneal haze. The eyes were examined at 4, 24, and 48 hr. One animal, having received undiluted toxin, was re-examined weekly for a period of 3 months.

**Results**

**Hemolysin titers of strains.** A comparison of hemolysin titers in strains of proven and of undetermined virulence is illustrated in Fig. 1. These results show that all strains of proven virulence had high titers (1:8 or greater) of hemolysin whereas those of undetermined virulence had a wide range of titers, with most strains (65%) having a titer less than 1:8.

**Effects of intracorneal injection of purified hemolysin.** Fig. 2 shows the effects of injection of 0.05 ml of a 10-fold dilution of the purified hemolysin preparation. Extensive corneal opacification and white discharge were observed 24 hr...
Fig. 2. Appearance of rabbit eye 24 hr after intracorneal injection of purified *P. aeruginosa* hemolysin.

after the injection and were intensified at 48 hr. Histological examination of corneas from animals sacrificed 48 hr after injection showed extensive leukocytic invasion of the corneal stroma. In a rabbit given the same amount of undiluted lysin, the opacification was even greater and remained unresolved over the next several months during which the animal was observed.

**Discussion.** The extracellular, heat-stable hemolysin of *P. aeruginosa* has been studied by Liu, Berk, and Borderon et al. and would appear to be identical with the hemolytic glycolipid reported by Sierra. A heat-stable lysin located intracellularly has also been reported. The relationship between the intracellular and extracellular forms has not been clarified.

The finding (Fig. 1) that strains of *P. aeruginosa* of proven virulence are more hemolytic than strains of undetermined virulence suggests that the hemolysin elaborated by this organism plays a role in its pathogenicity. The finding of high titers in a few strains of undetermined virulence may reflect the fact that the group is of variable virulence, some possessing a high potential virulence which was not, however, manifested in the environment from which they were isolated. In another study of correlation between characteristics of strains of *P. aeruginosa* and their virulence, Kawaharajo and Homma found that only those strains positive for protease and elastase production were able to cause lesions of incised mouse corneas.

Further confirmation of the role of the hemolysin (which is probably, like most “hemolysins,” actually a cytolysin capable of lysing various cell types) in corneal pathogenesis is provided by the observations of corneal damage produced upon intracorneal inoculation of purified lysin (Fig. 2). These effects which we observed on injection of *P. aeruginosa* cytolysin are similar to those reported by us to result from injection of purified cytolysin from the pneumococcus. Another worker has postulated that the ability of strains of *P. aeruginosa* to initiate respiratory infection is related to the degree of hemolysin production, on the basis of an observed correlation between hemolysin content and toxic activity against macrophages.

It is possible that rather than directly damaging host tissue (or perhaps in addition to that mechanism), the *P. aeruginosa* cytolysin may act indirectly on the leukocytes which were observed to accumulate at the site of injection. Lysis of the leukocytes, or stimulation of these cells to release their lysosomal enzymes, could yield enzymes capable of degrading proteoglycan and collagen. Thus bacterial products and host cells would interact to produce pathological effects in addition to those produced by the direct action of bacterial enzymes. Since ulceration of the cornea was not observed in our experiments, we presume that the action of other toxic components of the organism (e.g., proteases, exotoxin) is required for the ulceration which is observed during the course of *P. aeruginosa* infections.
It is, of course, also possible that the high hemolysin content which we have observed in virulent strains was only coincidental and that the inflammation caused by its injection is not part of the natural disease process. We plan to evaluate further the specific role of a cytolytin in corneal disease by comparing the course of infection in eyes inoculated with hemolysin-producing strains with that observed upon inoculation with hemolysin-negative (isogenic) mutants.

From the Departments of Microbiology and Ophthalmology, Tulane University School of Medicine, New Orleans, La. This study was supported by grant EY 00424 from the National Eye Institute. Submitted for publication Aug. 15, 1977. Reprint requests: Mary K. Johnson, Department of Microbiology, Tulane University School of Medicine, New Orleans, La. 70112.

Key words: Pseudomonas aeruginosa, hemolysin, corneal infections

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