EXPERIMENTAL *PSEUDOMONAS* KERATITIS

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INTRODUCTION

PSEUDOMONAS AERUGINOSA IS NOW A COMMON, AND PROBABLY THE MOST IMPORTANT, cause of bacterial infection of the cornea.[1-3] Laibson[1] noted that the "keratitis produced by *Pseudomonas aeruginosa* is the most rapidly spreading and destructive bacterial disease the human cornea can be infected with, as well as the most disastrous. It is apparent that *Pseudomonas* has replaced *Staphylococcus, Pneumococcus,* and *Streptococcus* as the most common cause of severe necrotic corneal ulceration."

Pseudomonas keratitis has been a topic for research by myself and my colleagues. The first section of this study will chronologically describe serial, clinical, and histopathologic observations. Emphasis has been placed on scanning electron microscopy (SEM) and transmission electron microscopy (TEM) at various time periods after infection in a *Pseudomonas* keratitis model induced following superficial trauma in the rabbit. This model is probably more similar to the keratitis induced in humans than intrastromal induction models evaluated previously by ultramicroscopy.[4,5]

The second section of this study will clarify the roles of topical, subconjunctival, periocular, and systemic therapy, either individually or as adjunctive treatment. Comparative evaluations will be made by using a superficial trauma-*Pseudomonas* keratitis model in rabbits.

HISTORICAL INFORMATION

HISTORY OF ORGANISM

Most *Pseudomonas* species do not infect human beings, but some are important opportunistic pathogens that infect patients with impaired local or systemic host defenses. The species that is most important and most frequently associated with human disease is *P aeruginosa.*[6]

The early history of *P aeruginosa* is interesting because the pigment products of the organism were recognized long before the organism itself. Physicians knew that blue-green pus in a wound or on a surgical dressing was a sign of a poor prognosis, long before the organism had been isolated. The blue-green discolorations on surgical dressings were eventually associated with infection. Gessard first isolated the organism from these surgical dressings in 1882, and it was termed "*Bacillus pyocyaneus.*"[7] In 1889, Charrin demonstrated the pathogenicity of the organism in animals[8] and human beings[9] and already realized that organism-free filtrates played a role in pathogenesis.

FACTORS IN PATHOGENESIS OF *PSEUDOMONAS* INFECTIONS

As in any bacterial infection, the severity of the disease produced depends on factors related to the character of the microorganism, its interaction with host tissue, and the host cell responses.

ROLE OF ORGANISM

Glycocalyx ("slime envelope").—The importance of the glycocalyx was ignored until recently because strains of bacteria grown in the laboratory do not require it, and therefore do not expend the energy to produce it. Since it disperses readily in liquid media, it is not a true capsule. However, it is a morphologically distinct, well-defined layer, frequently felt-like in certain species, surrounding an individual cell or a colony of bacteria. It promotes adhesion between bacteria as well as adhesion of the bacteria to a tissue or cell surface.[10,11] In 1978, Costerton et al[12] stated that "Much as adhesion to a rock in a stream benefits the bacteria in a stream, adhesion to a particular tissue surface may play an important role in the pathogenesis of certain infections." Although this has not yet been proved, bacterial infection of certain surfaces, and possibly the cornea, may begin with the specific adhesion of the bacterium to the wounded surface before tissue invasion occurs. The bacterial cell-to-cell adhesion and the bacterial cell adhesion to a particular animal tissue or cell surface then provides an environment for bacterial toxin and enzyme diffusion that may be necessary in early bacterial invasion of certain tissues.

In patients with cystic fibrosis, *P aeruginosa* is an important cause of pneumonia. A number of mucoid strains of *P aeruginosa* that have a glycocalyx have been isolated from the respiratory tracts of these patients and studied, as has phagocytosis inhibition.[13] Inhibition of phagocytosis is considered another important role of the glycocalyx or slime envelope. By promoting bacterial cell-to-cell adhesion, aggregates or microcolonies are

formed that resist phagocytosis by host leukocytes. The role of the slime envelope as a toxin has been unclear.[11,14]

Extracellular Products.—The importance of extracellular products in the pathogenesis of *Pseudomonas* infections was appreciated as early as 1889, (as mentioned previously) when Charrin established the pathogenicity of *P aeruginosa* in animals by demonstrating the toxicity of cell-free filtrates of cultures of the organism.[8] However, until the last few decades, the work was either forgotton or ignored, and pathogenesis was traditionally ascribed mainly to a endotoxin.[15]

An understanding of the mode of pathogenesis of systemic *P aeruginosa* infections has progressed greatly since it was realized that endotoxin was of less importance and that the important histopathology of the infection could be produced solely with the extracellular products of *P aeruginosa*. In systemic infections, the most important factor reported has probably been the "lethal" exotoxin A. Other toxins contribute to pathogenesis in systemic infections, but, because of their localized activity, they probably play a less important role systemically. However, their localized activity in corneal infection appears to be more significant, as noted later.

The extracellular products are as follows:

1. *Pigments*—The pigments of *P aeruginosa*—pyocyanine (a phenazine pigment) and fluorescein—have been the best known products of the species. Contrary to traditional thinking, they probably have not played an important role in pathogenicity. The significance of the pigments in pathogenicity has probably resulted because α-oxyphenazine, a fraction of the phenazine pigment, contains an antibiotic, pyocyanase, with an effect that long preceded the use of sulfa drugs and penicillin in Europe. Therefore, the significance of legendary pigments produced by *P aeruginosa* may be in the inhibition and replacement of other flora by *P aeruginosa*.[16]

2. *Proteases*.—Proteolytic enzymes secreted by *P aeruginosa* are termed proteases. They represent a mixture of enzymes that will break down various proteins and whose functions vary significantly. Usually, two to three proteases can be separated in the supernatant of a *P aeruginosa* strain culture.[17] They generally liquefy gelatin and milk, and may dissolve elastin.[18] Also, many strains elaborate proteases that function as pseudocollagenases which can attack terminal peptides of native collagen and liberate amino acids or peptides.[19-21] However, these are unlike the classic collagenase of *Clostridium histolyticum*, which characteristically cleaves collagen with the liberation of hydroxyproline.[22] Proteases of *P aeruginosa* that affect collagen should probably be thought of as a group of nonspecific collagenases.[16]

Proteolytic activity was prominent in the harvest media of strains evaluated by Brown et al,[23] and intralamellar injection liquified rabbit cornea. The activity was inhibited by NA_2 EDTA. They demonstrated that the enzyme lacked significant collagenolytic activity, but was capable of degrading proteoglycans in vitro. Gray and Kreger[4] also reported that proteoglycans were broken down and collagen appeared intact in their observations of infected rabbit cornea at 24 hours after infection, but the evidence was indirect. The possible role of host enzymes in corneal damage was noted by the previously mentioned investigators. This premise was further explored by Kessler et al,[24,25] who demonstrated that proteoglycanase activity can be derived from either host cell activity or from a purified enzyme from the organism. They concluded that corneal destruction induced by *P aeruginosa* depended not only on protease but also on host-derived enzymes that are capable of breaking down both collagen and proteoglycans. The role of the host response will be developed in the next section.

Elastase is also one of the proteases of *P aeruginosa*,[26] which has been shown to cause corneal destruction in mice.[27] However, it has recently been demonstrated, using *P aeruginosa* mutants without elastase, that elastase activity does not appear to be essential for corneal destruction in *P aeruginosa* corneal infections.[28]

3. *Hemolysins*.—Hemolysins, primarily phospholipases, probably play a significant role in the pathogenesis of certain *P aeruginosa* infections. In pneumonia caused by *P aeruginosa* they interfere with a surfactant that normally coats alveoli, thereby reducing surface tension and causing atelectasis, and they may cause necrosis of lung tissue.[29] Johnson and Allen[30] demonstrated that intracorneal injection of purified hemolysins from *P aeruginosa* caused corneal opacification and leukocyte infiltration without corneal ulceration. In addition, higher titers of extracellular hemolysins were found in strains that were virulent for the cornea than in less virulent strains.

4. *Exotoxin*.—Although other extracellular products have some importance in pathogenesis in systemic infections, the virulence of *P aeruginosa* in such infections is now thought to correlate mainly with the ability of the particular strain to produce three exotoxins (A, B, and C) that are lethal for mice and dogs and cause hypotensive shock in monkeys.[6] The lethal toxin, exotoxin A, has recently been isolated. It is an extremely powerful inhibitor of cellular protein synthesis, causing necrosis of liver and kidney tubules, hemorrhagic lungs, blood macrophage death, and impairment of phagocytosis, the latter being the first line of normal defense in *Pseudomonas* infections.[16,31] The NAD-ase activity of exotoxin A inhibits the

synthesis of protein at the ribosomal level, and the enzymatic activity appears to be identical to that of diphtheria toxin fragment A.[32]

Iglewski et al[33] recently showed that intrastromal corneal injections of purified exotoxin A from *P aeruginosa* killed epithelial and endothelial cells, and presumably stromal keratocytes, causing corneal edema and clouding in the first 24 hours. Collagen destruction and ulceration occurred later when polymorphonuclear leukocytes (PMNs) had also invaded. Ohman et al[28] also reported that exotoxin A is not required to initiate corneal damage, although it does play a role in the severity of resultant corneal damage, and possibly in the maintenance of corneal infection.

5. *Endotoxin*.—As previously described, systemic virulence and pathogenesis have been shown to be due primarily to the extracellular products of *P aeruginosa*, while the role of endotoxin or cell wall lipopolysaccharide is probably insignificant in systemic disease.[16] However, it does appear to play a role in corneal disease. Purified endotoxin injected into rabbit corneal stroma produced a ring of PMNs in which properdin and C_3, but not immunoglobulin, were found. This indicated to the authors that endotoxin may stimulate the alternate complement pathway in the cornea.[34]

ROLE OF HOST RESPONSE

The phagocytic cell, primarily the PMN, is the principle host defense against *Pseudomonas*. In systemic infections, humoral immunity (specific antibody or opsonins) appears to provide a second line of defense. Cell-mediated immunity is a major defense against intracellular organisms such as *Mycobacterium* and *Listeria*, but its role in *Pseudomonas* infection has not, to my knowledge, been adequately studied.[31]

In keratitis due to *P aeruginosa*, PMNs play a significant role in host cell defense as well as in corneal destruction. In an interesting experiment to evaluate the role of the PMN in host cell defense, animals were made neutropenic with x-radiation, and there was a relative paucity of PMNs in the corneal stroma compared with corneas similarly infected by *P aeruginosa* in nonneutropenic animals. Quantitatively, the study showed that significantly higher titers of bacteria were present in neutropenic animal corneas, using three *P aeruginosa* strains.[35] Although the PMN is important in host defense, it has at the same time been implicated in the destruction of the cornea collagen matrix[5,36,37] or ground substance.[24]

In the alkali-burned rabbit cornea, it has recently been shown that stromal ulceration and collagen degradation can be prevented by mini-

mizing PMN infiltration with a glued-on contact lens.[38] It would be interesting to study the latter result in experimental *Pseudomonas* keratitis, using an experimental model where superficial trauma, rather than experimental intrastromal inoculation, was employed to induce infection.

Mondino et al[39] reported that complement may contribute to PMN infiltration of the cornea and to destruction of invading organisms in *P aeruginosa* infections, with *Pseudomonas* endotoxin influencing the sequence of events and responsible for the peripheral "corneal ring."

Jones[40] recently reviewed the pathogenesis of bacterial and fungal keratitis. He emphasized that not only are host-cell PMN lysosomal enzymes (such as collagenase and proteoglycanase) implicated in the pathogenesis of corneal destruction in *Pseudomonas* keratitis, but that oxidizing products of a metabolic oxidative phenomenon occurring when microorganism(s) and phagocyte contact have also been implicated in microbial killing and damage to host tissue. This mechanism may play a role in microbial killing in corneal infections, which will be discussed further.

Berman[41] has also reported that PMNs play an important role in degrading corneal matrix, probably through PMN extracellular release of collagenase, elastase, and cathepsin G, effecting collagen degradation. However, although various PMN granule enzymes in rabbits and human beings have been recently identified, the characterization and functional significance of the various PMN rabbit and human granule enzymes have not yet been clarified.[42]

MORPHOLOGY AND CHARACTERISTICS OF ORGANISM

P aeruginosa is a slender, gram-negative, highly motile rod. Its dimensions vary in length from 1 to 3 μ and from 0.5 to 1.0 μ in width. The variation in dimensions is due to the extracellular material coating, the glycocalyx (slime envelope capsule), which has previously been discussed.[43] There is no membrane associated with the outermost regions of the envelope.

It is one of the easiest bacteria to culture, growing on a variety of media and tolerating a wide temperature range. It is an obligate aerobe. Some experienced microbiologists, with a hanging drop preparation, can make a diagnosis of *P aeruginosa* under the light microscope, mainly because of its extreme motility, according to M. Okumoto, MA, (personal communication, August 1970).

P aeruginosa has been isolated from hexachlorophene soaps, antibiotic solutions, and certain quarternary ammonium compounds. It is ubiqui-

tous—abundant in soil and water, found on skin, in saliva, in the gastrointestinal tract, and wherever there is adequate moisture. It is a common contaminant of respiratory care equipment, cold water humidifiers, instruments, bedpans, floors, baths, and water faucets.[6]

P aeruginosa is more resistant to chemical disinfection than other vegetative bacteria, and resistant to most antibiotics and antimicrobial agents. Aminoglycosides such as gentamicin, tobramycin, and amikacin have been the most effective antibiotics used in the last decade. They are rapidly bactericidal for most *Pseudomonas* species by acting directly on the bacterial ribosome, where they inhibit protein synthesis. Clinically, resistance to gentamicin and tobramycin has been primarily a problem in the hospital, especially in burn units.[44-46] Increased resistance to aminoglycosides has not been a problem in surveys of organisms isolated from infections occurring outside the hospital environment.[47,48] However, in the last ten years, there has been an increased resistance to gentamicin and tobramycin in nosocomial infections. Amikacin has retained its value in this setting because of its resistance to most microbial enzymatic degradation; thus, it has been especially valuable in treating nosocomial infections.[46] Gentamicin remains effective in approximately 90% of isolated strains of *P aeruginosa*, tobramycin in approximately 95%, and amikacin in approximately 95%.[49]

EPIDEMIOLOGY OF SYSTEMIC AND CORNEAL INFECTIONS

Clinically, systemic infections caused by *P aeruginosa* occur primarily in persons with altered host defenses, ie, patients with thermal burns, cancer (especially acute leukemia), renal transplants, or cystic fibrosis, and in certain debilitated hospitalized patients. In patients with systemic infections, probably the most important host factors associated with susceptibility to infection with *Pseudomonas* are quantitative, and perhaps qualitative abnormalities of PMN function.[31]

In corneal infections, the most important host factor is usually a breach of the epithelial barrier function, which normally prevents potential bacterial invasion of the corneal stroma.[50] When there is a disruption of the corneal epithelium, either posttraumatic or in patients with certain corneal diseases such as bullous keratopathy or herpes keratitis (Fig 1A), organisms invade the corneal stroma from the tear film or as contaminants of foreign bodies (Fig 1B), irrigating solutions, fluorescein solutions,[51,52] cosmetics (Fig 1C),[53-55] and contact lenses or their solutions (Fig 1D). In my clinical practice, induction of *Pseudomonas* ulcers from wearing contact lenses, especially soft contact lenses, has become a common predis-

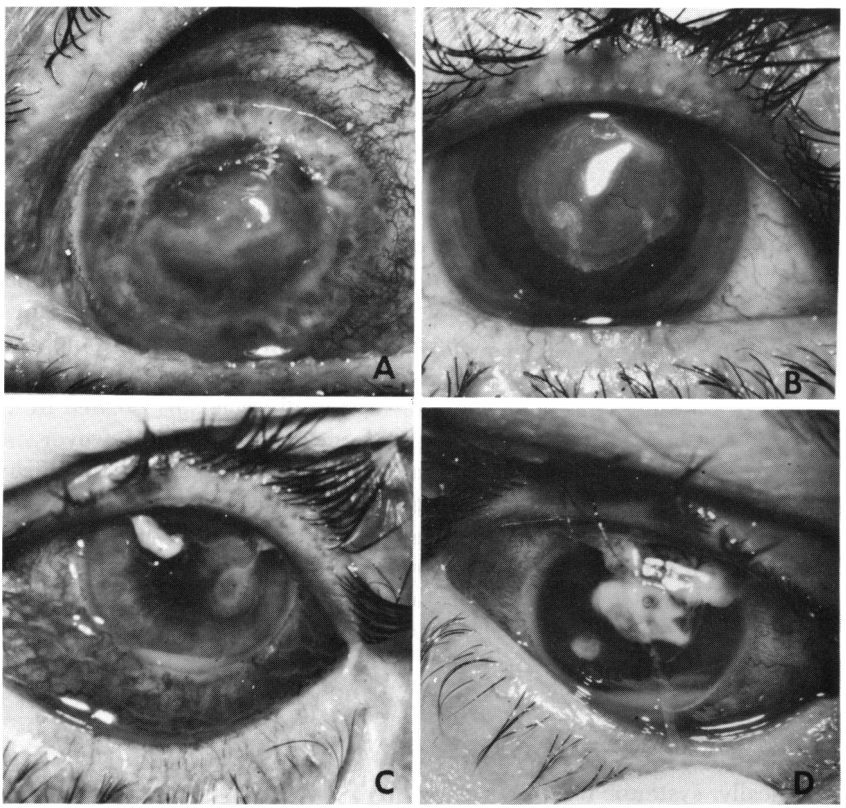

FIGURE 1
A: *Pseudomonas* keratitis superimposed on herpetic keratitis. B: *Pseudomonas* keratitis after fiberglass injury. C: *Pseudomonas* keratitis after corneal abrasion with mascara applicator. D: *Pseudomonas* keratitis after wearing soft contact lens.

posing factor in causing *Pseudomonas* corneal ulcers. *Pseudomonas* corneal ulcers have been reported with hard contact lenses,[56] but even more frequently with soft contact lenses.[57-60]

Patients with localized *P aeruginosa* corneal ulcers usually have no history of being given systemic or local corticosteroids or immunosuppressive drugs, and systemic host defenses are usually normal,[61] as opposed to patients with systemic *Pseudomonas* infections. However, cases of *Pseudomonas* keratitis have been reported in premature infants,[62] burn patients,[63] and comatose tracheostomized patients.[64] Also, the use of topical corticosteroids after removal of a corneal foreign body may increase the severity of *Pseudomonas* corneal ulcers in the human eye.[65]

HISTORY OF KERATITIS

Sattler,[66] in 1891, at the International Congress of Ophthalmology in Germany, was the first to report a case of an infected corneal ulcer occurring after injury, from which he isolated *B pyocyaneus*. In 1953, Spencer[67] reviewed the literature and noted that approximately 104 cases had been reported up to 1952. In the last decade it has become the most important cause of bacterial keratitis and, in some areas of the United States such as Florida[68] and Texas,[3] the most frequent cause of bacterial keratitis. In reviewing changing patterns in bacterial keratitis through the years, Ostler and co-workers recently pointed out that the percentage of *Pseudomonas* corneal ulcers treated at the University of California, San Francisco, increased from 12% (1952–1959) to 37% (1968–1975).[61] Thus, the trend has been to an increase in the incidence of *Pseudomonas* keratitis since the 1950s, although the increase may be due in part to improvements in diagnostic techniques.

P aeruginosa is the most common species of the genus *Pseudomonas* to cause a hypopyon corneal ulcer, although other species such as *Pseudomonas cepacia, Pseudomonas maltophilia, Pseudomonas stutzeri,* and *Pseudomonas acidovorans* may rarely be corneal pathogens.[69]

CLINICAL APPEARANCE OF KERATITIS

The appearance of *P aeruginosa* has impressed clinicians for generations. In 1908, Axenfeld[70] stated that although it was a rare cause of bacterial keratitis, he was impressed with the clinical features of the *B pyocyaneus* corneal infection.

> The cases . . . were obviously due to a very virulent bacteria; in them extensive necrosis of the cornea was very evident, and the base of the ulcer was covered with the necrotic debris of the corneal lamellae. The toxins from the cornea so act on the tissues around that the conjunctiva bulbi may swell and given the impression of a panophthalmitis before the deeper parts of the vitreous are suppurating.

P aeruginosa is no longer a rare cause of bacterial keratitis, and two generations later Jones,[71] another experienced clinician, has accurately described often-associated clinical features characteristic of many untreated *P aeruginosa* corneal ulcers that may heighten a clinician's suspicion.

> The most pathognomonic ulcer is that produced by *P aeruginosa*, particularly if there is diffuse liquefactive necrosis, yellow-green purulent material adherent to the surface, and a history of rapid evolution (within 72 hours following trauma). Early focal ulcers also may be distinguished by the mucoid necrotic stroma and

irregular borders of infiltration beneath the epithelium. There is typically a "ground glass" appearance to the remainder of the cornea, with loss of transparency over a large area away from the ulcer. The infection may progress rapidly to produce a ring abscess and corneal perforation.

However, although there may be characteristic clinical features associated with certain forms of bacterial keratitis, as demonstrated in Figure 2, it is not possible to make a specific, reliable, etiologic diagnosis by just the clinical appearance of an ulcer.[71] This would be a special problem for the clinician, especially when there has been previous corneal disease, previous surgery, or previous antibiotic or corticosteroid therapy.

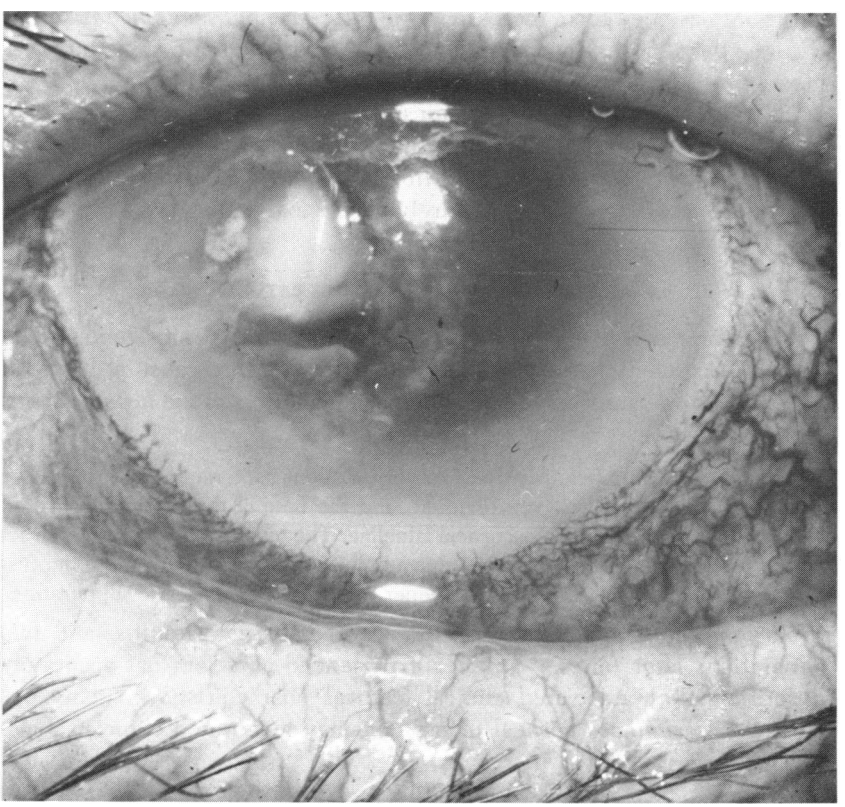

FIGURE 2
Typical *Pseudomonas* corneal ulcer following problems during wearing of aphakic soft lens. Note adherent purulent material and characteristic "ground glass" gray appearance over large area of cornea, well away from actual ulcerative infiltrates.

LABORATORY EVALUATION OF CLINICAL CORNEAL ULCERS

The most important step in the management of bacterial or fungal keratitis is the initial corneal scraping for culture and smear.[71,72] This procedure should be done for all corneal ulcers suspected of being bacterial or fungal in origin before antibacterial or antifungal therapy is initiated. Once antibiotic therapy has been started, the yield from a microbiologic workup is decreased significantly, even though the infection is progressing. Bacteriologic cultures and smears are significant only when the result of the culture or smear is positive. If the results of cultures or smears or both are negative, after adequate scrapings, a bacterial infection is not absolutely ruled out. Repeated scrapings should then be done.

Cultures, rather than smears, are generally the more sensitive means of establishing a diagnosis, although both should be used in the primary workup. There is some difference of opinion by experienced clinicians with regard to the relative importance one should place on the smear when deciding on initial treatment.[3,73] I believe the smear frequently gives prompt, important information that may be helpful in deciding on the emphasis of immediate therapy. However, I never exclude initial gram-negative therapy for the first 24 hours because of the absence of gram-negative organisms on the initial smear. The number of organisms seen in smears from scrapings of *Pseudomonas* corneal ulcers frequently are not as numerous as with scrapings from gram-positive ulcers, according to M. Okumoto, MA (personal communication, August 1970); also, with less contrast, they may be more difficult to distinguish than gram-positive organisms. The limulus lysate assay, which is extremely sensitive in detecting gram-negative endotoxin, has recently been evaluated and may be a useful adjunct in the initial diagnostic workup of bacterial keratitis resulting from gram-negative organisms.[68,74,75] As with the smear, however, it may not detect the presence of gram-negative organisms in some corneal infections caused by such organisms.[74] Its adjunctive value awaits further evaluation.

The details of the workup of a suspected bacterial corneal ulcer are nicely detailed elsewhere.[71,72] A schematic summary of the typical clinical laboratory workup done by myself and my colleagues in our unit is seen in Figure 3. However, I wish to emphasize certain points:

1. A Kimura spatula is ideal for conjunctival scrapings but should be modified for corneal ulcer scrapings. (The spatula is modified by tapering it to a narrow tip and abrading the platinum surface with a fine carborundum hone stone). The narrowed spatula is more useful for ulcer craters of all sizes and is able to hold corneal specimens much better than the standard Kimura spatula.

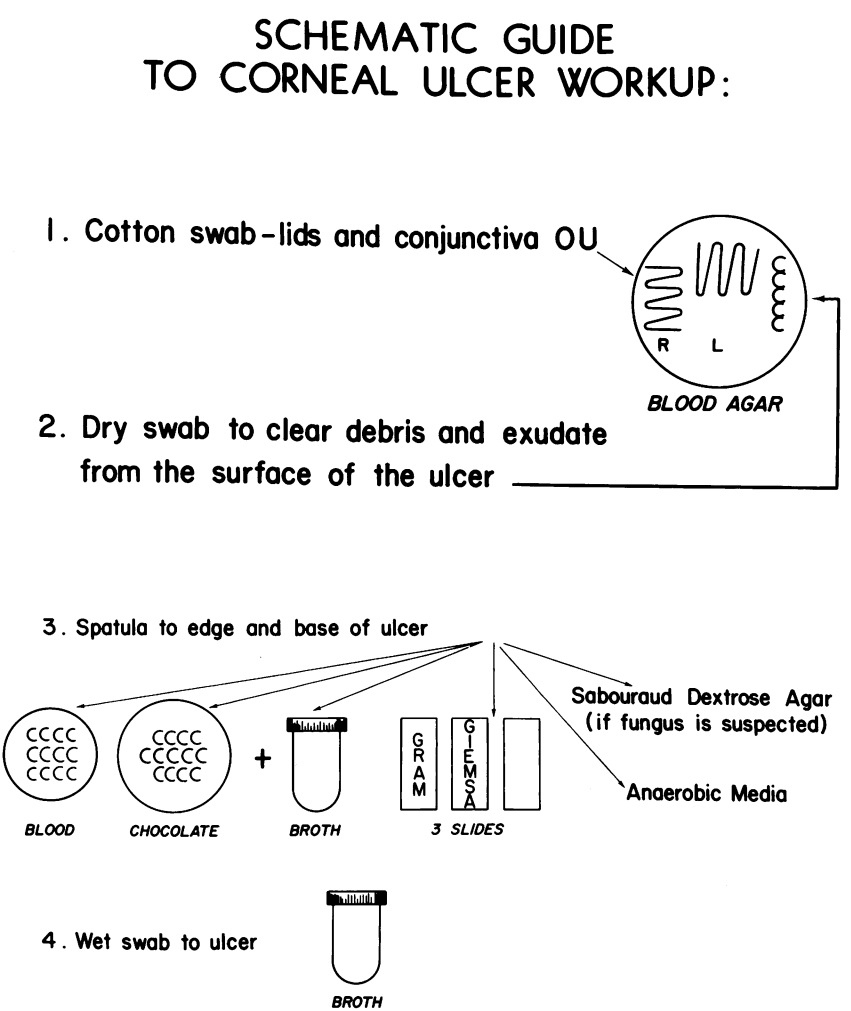

FIGURE 3
Schematic workup of corneal ulcer.

2. Multiple scrapings are taken, and microbiologic media are inoculated first. Cultures are more specific, and the yield is usually higher unless the patient has previously been taking antibiotics. The smear may then assume more importance, although Gram-staining characteristics may be changed after antibiotic use.

3. Multiple C-streaks are made on solid media. Any growth on a C-streak is regarded as specific, whereas any growth off of a C-streak is interpreted as probable contamination.

BACKGROUND FOR EXPERIMENTAL STUDIES

ELECTRON MICROSCOPIC STUDIES OF *PSEUDOMONAS* KERATITIS

Only a few ultrastructural studies of corneal infection caused by *P aeruginosa* have been published.[4,5,76]

HUMAN ELECTRON MICROSCOPIC STUDY

The first study was reported by Van Horn et al,[76] in 1973, on a pair of human corneas obtained at autopsy. The patient had been comatose for seven weeks with bilateral *Pseudomonas* keratitis that had been treated with gentamicin. Cellular infiltration was seen in both corneas, and extensive degradation of collagen was described in one cornea that had perforated. At the stage of observations, no organisms were found in the previously antibiotic-treated cornea. The authors concluded that they could not determine which factor—the organism, granulocytes, or damaged epithelium—played the most important role in collagen breakdown.

EXPERIMENTAL KERATITIS ELECTRON MICROSCOPIC STUDIES

Live *P aeruginosa*.—TEM studies of experimental *Pseudomonas* keratitis have been published, using experimental models in which live organisms[4,5] or their products[33] were injected intrastromally. Using the intrastromal infection technique, organisms or products are not confined to a few millimeters of the zone of infection, but the injected material immediately diffuses throughout the cornea and dissects intralamellarly to the peripheral limbus (DL Van Horn, PhD, unpublished data). Also, this method of induction differs from induction in human beings, which frequently follows superficial trauma or contaminated superficial foreign bodies. Early ulceration occurs, and inflammatory cell infiltration initially may be from the tear film early in the disease process, compared with intrastromal challenge. However, the studies reported, using the intrastromal injection model, have relevance and provide information concerning concepts that may play a role in corneal damage in *Pseudomonas* keratitis.

Gray and Kreger[4] examined the rabbit cornea and reported on observations 24 hours after intracorneal injection of *P aeruginosa*. At that time

they noted epithelial degeneration, loss of keratocytes and endothelium, extensive corneal edema, and dispersal of ultrastructurally normal collagen fibrils. The authors concluded that a loss of corneal proteoglycan weakened the cornea and led to later descemetocele formation and corneal perforation by the intraocular pressure.

Van Horn et al[5] reported on electron microscopic observations in guinea pig corneas. They observed loss of corneal cells only when extensive degradation of PMNs had occurred 24 and 48 hours after intrastromal injection of live bacteria. A mild infiltration of PMNs and no degradation of collagen was seen after injection of only heat-killed bacteria. Collagen breakdown was first noted at 24 hours after injection of live bacteria. Ultrastructural evidence of collagen breakdown included loss of intact collagen fibrils, accumulation of amorphous electron-dense material, and tactoid formation.[77] The ultrastructural changes reported in these guinea pig corneas infected with *P aeruginosa* were remarkably similar to the ultrastructural changes in rabbit corneas described by Rowsey and Nisbet[36] after intracorneal injection of concentrated PMN lysosomal preparations. They noted that corneal collagen fibrils were replaced by amorphous deposits of electron-dense material and tactoid formation, as in in vitro studies of collagen degradation where tactoid formation occurred, and that this was indicative of proteinaceous breakdown products of collagen.[77] Similar unpublished studies have recently been carried out in rabbits after intracorneal injection of viable *P aeruginosa*.[37] Electron microscopy revealed accumulations of small electron-dense granules in association with collagen fibrils and degranulating PMNs, but no tactoid formation or accumulation of amorphous electron-dense material, as seen in guinea pig cornea. As the keratitis progressed, the electron-dense granules were found in larger areas of stroma and may have represented products of collagen or proteoglycan breakdown or both. The authors noted that similar accumulations of electron-dense particles were also described in unpublished electron micrographs by Gray and Kreger. No evidence of corneal damage was found in association with bacteria alone. The corneal damage lagged considerably behind the rapid increase in the number of *Pseudomonas* organisms and was closely associated with the infiltration and degranulation of PMNs.[37]

Approximately four days after infection, undigested intact *Pseudomonas* organisms were noted by the investigators in completely degranulated PMNs. They thought this might be an explanation for persistence of some *Pseudomonas* organisms despite therapy[78] and for relapses reported by some authors.[79,80] They also noted that some bacteria in corneal stroma were encompassed with a glycocalyx (slime envelope).

Although not discussed by the authors, the glycocalyx observed with some strains of *P aeruginosa* in noncorneal infections has been reported to function in adhesion and also in inhibition of phagocytosis by the PMN. Phagocytosis has been shown to be the main host-defense mechanism in systemic *Pseudomonas* infections[31] and this may also be the case in corneal infections, as the present study will show.

P aeruginosa Products.—Iglewski and colleagues[33] reported on ultramicroscopy after intrastromal injection of purified *Pseudomonas* exotoxin A into rabbit cornea. They observed infiltration with PMNs and death of epithelium, keratocytes, and endothelium, with collagen bundles ultramicroscopically intact at day 3.

Investigative work has been done in attempts to clarify our concepts and understanding of *Pseudomonas* keratitis. I will report on sequential electron microscopic observations in experimental *Pseudomonas* keratitis, beginning immediately after infection, and using another experimental model that may be more similar to many cases of human keratitis than the intrastromal injection models previously used for electron microscopic evaluation of *Pseudomonas* keratitis. SEM studies also have not previously been reported in experimental *Pseudomonas* keratitis. In this study, SEM will be correlated with TEM observations and with clinical observations of the infectious keratitis following superficial trauma at multiple times after infection.

ANTIMICROBIAL TREATMENT OF *PSEUDOMONAS* KERATITIS

BACKGROUND

In the past, a variety of therapeutic agents have been used in the treatment of *Pseudomonas* keratitis. Prior to 1940, the traditional vigorous therapy for a *Pseudomonas* corneal infection may have included hot packs, atropinization, irrigation, antiseptics, Saemish incision, and cauterization of the cornea, all of which were usually unsuccessful in preventing loss of the eye.[81-83] Joy,[83] in his thesis as an American Ophthalmological Society candidate, reported some therapeutic success in his trials with sulfapyridine sodium. He reported that if oral sulfapyridine was administered prophylactically or within six hours of onset of infection, it had a beneficial effect on the course of the disease. Prior to 1950, however, the degree of virulence of the organism was probably more responsible for the ultimate outcome of the corneal disease than any particular form of therapy.[84-91]

The management of *Pseudomonas* keratitis began to change around 1952 when reports of clinically significant therapeutic success labeled

polymyxin B sulfate the drug of choice.[67,92-94] Colistin (polymyxin E) also proved effective in England,[95] and became another sound therapeutic choice in the United States in the 1960s.[96-100]

AMINOGLYCOSIDE THERAPY

In the last decade, the aminoglycoside gentamicin has proved effective in the treatment of *P aeruginosa* keratitis,[78,101-105] and has replaced polymyxin B and colistin in the initial management. Although carbenicillin may act synergistically with gentamicin in vitro and has been recommended for use along with gentamicin, no synergism was seen when evaluated in experimental *Pseudomonas* keratitis.[103]

Tobramycin, a newer aminoglycoside, has also proved extremely effective in the treatment of *P aeruginosa* keratitis.[78,104,106-110] Tobramycin is similar to gentamicin in antimicrobial activity and toxicity and is reported to be two to four times more active in vitro by weight, as compared with gentamicin, against *P aeruginosa*.[111] It has been shown to be effective against some gentamicin-resistant strains,[112] and approximately 50% of *P aeruginosa* organisms resistant to gentamicin remain sensitive to tobramycin.[46] However, there has been no evidence to date that tobramycin has been significantly superior clinically to gentamicin in the treatment of *Pseudomonas* keratitis.

The current preferred drugs for the initial treatment of suspected gram-negative rod keratitis and *Pseudomonas* keratitis are either gentamicin[2,3] or tobramycin.[73] When a strain is resistant to gentamicin and tobramycin, amikacin may be tried. Most acquired resistance to aminoglycosides has occurred from acquired microbial enzymatic inactivation in the bacterial membrane or near the site of drug transport. Amikacin is a new semisynthetic aminoglycoside that is resistant to inactivation by most bacterial enzymes,[46] and has had a good therapeutic effect against *P aeruginosa* keratitis.[104,105,113] Colistin or polymyxin B may also be useful therapeutic alternatives.[114]

ROUTES OF ADMINISTRATION

The routes of antibiotic administration usually recommended include topical, subconjunctival, and occasionally, systemic. Systemic antibiotics have not been routinely recommended in the treatment of infected corneal ulcers.[2,73] Only low concentrations of gentamicin can usually be achieved in the cornea and aqueous after systemic administration, even in inflamed eyes,[115] and this has been true with other antibiotics.[116] Litwack et al[117] found good penetration of gentamicin in the secondary aqueous of rabbits, but only with doses much too large for human beings. Some

clinicians routinely recommend concomitant systemic antibiotics in moderate-to-severe corneal ulcers.[3,71,118] Does their use, especially of the aminoglycosides gentamicin and tobramycin, warrant the possible systemic toxicity? I believe systemic antibiotics should be used as concomitant therapy along with local antibiotics in corneal infections caused by *Neisseria gonorrhoeae* and *Hemophilus* in which other noncorneal tissues are frequently involved; in imminent or perforated corneal ulcers; in corneal ulcers with associated scleral infiltration or endophthalmitis; and, in some cases, as adjunctive therapy when an ideal local antibiotic regimen cannot be utilized. An evaluation of the role of systemic gentamicin in *Pseudomonas* keratitis will be made in a portion of the following experimental therapy studies.

Sloan and associates[119] demonstrated that topical aminoglycosides provided high therapeutic levels of antibiotic in the aqueous of infected eyes when high topical concentrations of antibiotics were given at 15-minute intervals. The authors of this excellent study compared frequent topical administration of the fortified gentamicin solution (20 mg/ml) with subconjunctival gentamicin and continuous-lavage gentamicin. Extremely high therapeutic levels of gentamicin were attained by all routes. They concluded that since similarly high concentrations of gentamicin can be attained in the infected eye by the less traumatic means of frequent topical drops, topical medication may prove more logical and just as effective a form of therapy as subconjunctival antibiotics. Baum and associates[120] reported significantly higher concentrations of antibiotic in the infected and inflamed cornea after adminstration of subconjunctival gentamicin than after topical gentamicin, which seems to conflict. However, they compared a topical therapy regimen using one drop of gentamicin (3 mg/ml) every 30 minutes with subconjunctival injection of gentamicin (20 mg).

The ultimate test of experimental antibiotic therapy, however, is the effect of the antimicrobial when various routes on the bacterial population itself in the infected cornea and quantitative microbiologic assays are used. Recently, Davis and colleagues demonstrated that topical aminoglycosides significantly reduced the number of viable *P aeruginosa* organisms in experimental keratitis.[78,104,105,108-110] The work was confirmed by others using similar techniques.[121]

Variables in the use of topical aminoglycoside antibiotics have recently been evaluated by using microbiologic assay of corneal organisms after treatment. When employing higher concentrations of antibiotics topically, there was no therapeutic advantage in using a dosage interval of less than 30 minutes; that is, there was no significant difference between

topical treatment with tobramycin every 15 minutes or every 30 minutes. However, treatment every 15 minutes or every 30 minutes was significantly better than treatment every 60 minutes or 120 minutes.[78] Other important factors that enhanced topical therapy were as follows: (1) removal of corneal epithelium (when lower concentrations were used), (2) increasing the concentration of antibiotic, and (3) starting therapy earlier in the course of infection.[108] Initial intensive therapy with high concentrations of topical antibiotic killed 99.9% of the organisms in the first 24 hours. The remaining bacteria declined gradually over several days, and less intensive therapy probably would have been needed.[109]

Is subconjunctival therapy usually necessary as adjunctive treatment in *Pseudomonas* keratitis when frequent, high concentrations of a topical aminoglycoside can be administered? As mentioned previously, Sloan et al[119] questioned the logic of traumatic subconjunctival therapy when intensive topical therapy could be used, considering the high concentrations that could be achieved in infected corneas. When intensive topical antibiotic therapy has been instituted, quantitative objective proof of the efficacy and additional benefit of subconjunctival antibiotics has not, to my knowledge, been reported in the literature. This is important, especially since subconjunctival antibiotic therapy has certain disadvantages: patient apprehension, more ocular inflammation, more pain than topical therapy, and a risk of intraocular administration (although rare, important). Recently, Davis and associates[122] reported in a comparative study of experimental *Pseudomonas* keratitis in rabbits and guinea pigs that a highly concentrated dose of topical tobramycin (20 mg/ml) administered every 30 minutes was significantly more effective than subconjunctival therapy with tobramycin (20 mg) in eliminating *Pseudomonas* from the cornea. Also, in a therapy trial with combined topical tobramycin and subconjunctival tobramycin, subconjunctival therapy did not improve the efficacy of intensive topical tobramycin in killing organisms in the treatment of *Pseudomonas* keratitis.

A recent paper presented at the 1980 national meeting of the Association for Reseach in Vision and Ophthalmology (ARVO) also compared topical and subconjunctival therapy by evaluating the number of residual viable organisms after drug delivery. Despite the administration of significantly more gentamicin by subconjunctival injection than by the topical route, the latter was significantly more effective in eliminating bacteria from the cornea, and the relative worth of subconjunctival antibiotics was questioned.[123] Although the models used were not human and animal studies are not directly applicable to human beings, the findings have relevance. The method of traumatization in the experimental model was

not superficial corneal trauma. In both studies *Pseudomonas* keratitis was induced by intrastromal inoculation of organisms, which is more dissimilar to the human model than superficial injury would be. The pharmacokinetics were likely to be different in the two models than in keratitis after superficial injury.

It seems clear that, with the use of higher concentrations of antibiotic given frequently, the topical route of antibiotic administration has been shown in multiple studies to be highly effective for suppressing bacterial growth in the cornea. Further experimental work, however, is warranted in an attempt to clarify the relative effectiveness of subconjunctival gentamicin and its role as adjunctive therapy to certain topical antibiotic regimens in the treatment of *P aeruginosa* corneal infections, ideally using the superficial injury model. This will be an important subject of investigations in the following studies.

MATERIALS AND METHODS

SEQUENTIAL ELECTRON MICROSCOPIC STUDIES

METHOD OF INFECTION

Male New Zealand White (NZW) rabbits weighing 2 to 3 kg were anesthetized by intramuscular injection of 1 ml of an equal mixture of ketamine hydrochloride (Ketaset) and xylazine (Rompun). Eyes were anesthetized by topical application of 0.5% proparacaine hydrochloride (Ophthaine). The corneas were traumatized by using the scratch model method (described in detail under "Keratitis Method Trials"), followed by instillation of three drops (at several-minute intervals) of an undiluted overnight broth culture of *P aeruginosa* (strain 107). Control eyes were traumatized in the same manner.

EXPERIMENTAL AND CLINICAL EVALUATIONS

The rabbits were killed with 5 ml of sodium pentobarbital administered intraperitoneally. The time intervals evaluated after infection were as follows: immediately after infection, 15 minutes, 30 minutes, and 1, 2, 4, 8, 16, 24, 72, and 96 hours. At least four infected eyes and two control eyes were evaluated at each time period. Clinical biomicroscopic evaluations were made at 8, 16, 24, 48, 72, and 96 hours, using a slit lamp (Haag-Streit 900).

METHOD OF FIXATION

The corneas to be examined by SEM and TEM were initially fixed before removal from the globe to maintain normal corneal curvature. This was important, especially for SEM studies. An intracameral injection of 0.05 ml of 2.7% phosphate-buffered glutaraldehyde was made using a 25-gauge needle on a tuberculin syringe, and two drops of glutaraldehyde were applied topically. The cornea, with a 3 to 4 mm scleral rim, was then excised and placed in several milliliters of 2.7% phosphate-buffered glutaraldehyde. After 24 hours of fixation, the corneas were halved perpendicular to the scratch with a razor blade.

For light histologic examination at 8, 16, 24, 48, and 72 hours after infection, the entire globe was removed and immediately fixed in 10% neutral buffered formalin.

TEM PROCEDURE

The half cornea for TEM was cut into 1.0 × 1.5-mm pieces and rinsed with a fresh change of slightly hypertonic phosphate buffer (pH 7.3) every ten minutes for at least 30 minutes. The specimens were postfixed in buffered osmium tetroxide for two hours and then quickly rinsed in three changes of deionized water. They were then dehydrated for 10 minutes each in 50%, 70%, 95%, and 100% (×2) ethanol and penetrated overnight in Spurr's low-viscosity embedding media. The prepared corneal pieces were then flat-embedded in fresh Spurr's low-viscosity embedding media at 70 C overnight. Thick sections were cut with an LKB pyramitome, placed on gelatin-coated slides, stained for 20 minutes at 65 C with methylene blue-Azure II and for five minutes at room temperature with basic fuscin. Selected areas were thin-sectioned with an LKB ultramicrotome, collected on a copper grid, and counterstained with saturated aqueous uranyl acetate for five minutes at 60 C, and with lead citrate for three minutes.

SEM PROCEDURE

The half cornea for SEM was rinsed with fresh phosphate buffer (pH 7.3) every ten minutes for at least 30 minutes. It was postfixed, rinsed, dehydrated, and penetrated following the procedure for TEM. After overnight penetration, the corneal surface was rinsed with hot acetone and placed in a vial in a 60 C hot-air oven for polymerization of the embedding resin. The specimen was mounted on an aluminum stub, sputter-coated with a gold-palladium alloy, and examined and photographed with a scanning electron microscope (AMR Model 1000).

LIGHT HISTOLOGY PREPARATION

The eyes were fixed in 10% neutral buffered formalin for a minimum of two weeks. Using an automatic tissue processor, the eyes were dehydrated in alcohol, cleared with chloroform, and infiltrated with paraffin. Serial sections (5 μ thick) were cut with a microtome (American Optical Co), and mounted on glass microscope slides. The sections were stained with Harris' hematoxylin-eosin phloxine counterstain using routine histologic methods.

EXPERIMENTAL KERATITIS/THERAPY TRIALS

STRAINS OF *PSEUDOMONAS*

The strains of *P aeruginosa* (107 and 115) used in this investigation were originally isolated from human corneal ulcers and were maintained by routine bacteriologic methods. Broth dilution, agar dilution, and disc diffusion antibiotic susceptibility tests were done using standard procedures.[124] However, the calcium and magnesium content of the Mueller-Hinton broth was adjusted to physiologic concentrations so that reliable in vitro results would be obtained, as recommended by Reller et al.[125] The strains were found to be susceptible to gentamicin in vitro, and both were virulent in the rabbit cornea.

KERATITIS METHOD TRIALS

The goal of this investigation was to find the experimental model that would yield a uniform and consistent keratitis with a high rate of infection by using surface trauma rather than intrastromal inoculation. Varied methods of surface trauma were used since, although intrastromal inoculation yields a uniform and consistent infection, the intrastromal model is less similar to the human model for infection following surface trauma.

Methods of Trauma and Infection.—Techniques to induce infectious keratitis were varied in 65 NZW rabbits weighing 2 to 3 kg. Twenty-one different methods of surface trauma and bacterial inoculation were evaluated (Table I). The instruments used for traumatizing the corneas included 25-gauge needles, corneal trephines, a chalazion curette, and a corneal rust ring remover. In one experimental group, the corneal epithelium was removed before scarification. The size of the area to be infected varied and ranged from a single 1.5-mm partial trephination to a limbus-to-limbus needle scratch. In the methods using a needle to induce keratitis, the area to be traumatized was outlined by lightly marking the corneal epithelium with a 3.0 or 5.0 mm trephine. The concentration of the

TABLE I: *PSEUDOMONAS* KERATITIS INFECTION TRIALS IN RABBITS

PSEUDOMONAS KERATITIS INFECTION TRIALS IN RABBITS	CLINICAL INFECTION RATE (HRS)		COMMENT
	24	48	
Needle Scratch (NS) Methods			
NS × 10, 5 mm: plate growth *Pseudomonas* + 2 drops *Pseudomonas* 10^{-2}	2/2	2/2	Mild keratitis
NS × 10, 5 mm: 2 drops *Pseudomonas* 10^{-2}	0/2	0/2	. . .
NS × 5, 5 mm: epithelium removed plate growth *Pseudomonas*	6/6	6/6	Infiltrates variable
NS × 5, 5 mm: plate growth *Pseudomonas* + 1 drop *Pseudomonas* 10^{-2} dilution	6/6	6/6	Mild keratitis
NS × 2, 3 mm: 1 drop undiluted *Pseudomonas*	7/8	7/8	Infiltrates variable
NS × 2, 3 mm: 2 drops undiluted *Pseudomonas*	8/8	8/8	Infiltrates variable
NS × 1, limbus-to-limbus: 1 drop undiluted *Pseudomonas*	7/7	7/7	Infiltrates variable
NS × 1, limbus-to-limbus: 2 drops undiluted *Pseudomonas*	7/7	7/7	Infiltrates variable
NS × 1, 5 mm: 1 drop *Pseudomonas* 10^{-2} dilution	2/6	2/6	. . .
NS × 1, 3 mm: 1 drop *Pseudomonas* 10^{-1} dilution	4/4	4/4	Mild infection
NS × 1, 3 mm: 1 drop *Pseudomonas* 10^{-2} dilution	1/4	1/4	. . .
NS × 1, 3 mm: 1 drop *Pseudomonas* 10^{-3} dilution	0/4	1/4	. . .
NS × 1, 3 mm: 1 drop undiluted *Pseudomonas*	12/12	12/12	Infiltrates variable
NS × 1, 3 mm: 2 drops undiluted *Pseudomonas*	8/8	8/8	Infiltrates variable
Trephination Methods			
Concentric trephinations 5.0, 3.0, 1.5 mm: plate growth *Pseudomonas* + 1 drop *Pseudomonas* 10^{-2} dilution	3/4	3/4	Infiltrates variable

TABLE I: CONTINUED

PSEUDOMONAS KERATITIS INFECTION TRIALS IN RABBITS	CLINICAL INFECTION RATE (HRS) 24	48	COMMENT
Ballantine sign, 3-mm trephine: plate growth *Pseudomonas* + 1 drop *Pseudomonas* 10^{-2} dilution	0/4	0/4	Stroma not cut
Multiple small 1.5-mm trephinations (3 rows-3 treph/row): plate growth *Pseudomonas* + 1 drop *Pseudomonas* 10^{-2} dilution	4/4	4/4	Infiltrates variable
1.5-mm trephine: 1 drop undiluted *Pseudomonas*	6/8	7/8	Infiltrates variable
1.5-mm trephine: 2 drops undiluted *Pseudomonas*	7/8	7/8	Infiltrates variable
Chalazion Curette (CC) Method			
CC, 5 mm: plate growth *Pseudomonas* + 2 drops *Pseudomonas* 10^{-2} dilution	9/10	10/10	Infiltrates variable
Corneal Rust Ring Remover Method			
Burr c̄ large foreign-body tip, 5 mm: plate growth *Pseudomonas* + 2 drops *Pseudomonas* 10^{-2} dilution	4/4	4/4	Infiltrates variable

Pseudomonas inoculum was also varied. In a few trials, the instrument used for scarification was dipped directly in an overnight culture of *Pseudomonas* grown on trypticase soy agar plates. Other trials used either an undiluted overnight culture of *Pseudomonas* in 10 ml of Mueller-Hinton broth, or a 10^{-1}, 10^{-2}, or 10^{-3} dilution of the overnight broth culture. Each drop of undiluted suspension contained approximately 5×10^8 viable organisms. Following traumatization of the corneas, either one or two drops of the *Pseudomonas* suspension were used alone or in combination with the direct plate growth of *Pseudomonas*.

Clinical Evaluation.—Slit-lamp observations were made at 24 and 48 hours after infection. The model yielding a uniform consistent keratitis with a high infection rate was also one of the most simple models—a 3-mm anterior stromal scratch using a 25-gauge needle, followed by

inoculation of one drop of an undiluted overnight broth culture of *Pseudomonas*. Using this scratch model, we have evaluated the frequency of infection in 41 rabbit eyes and have found that 97.5% (40/41) of the eyes became infected within 24 hours. Using the same method in 45 rabbit eyes, but inoculating with three drops of *Pseudomonas* given over several minutes (triplicate inoculation), we obtained a 100% (45/45) infection rate.

THERAPY TRIALS

Method of Infection.—*P aeruginosa* was inoculted into 10 ml of modified Mueller-Hinton broth[125] and incubated overnight at 37 C on a rotary shaker. Male NZW rabbits weighing 2 to 3 kg were anesthetized by intramuscular injection of 1 ml of an equal mixture of ketamine hydrochloride (100 mg/ml ketamine [Ketaset]) and xylazine (20 mg/ml [Rompun]). After topical application of 0.5% proparacaine hydrochloride (Ophthaine), the eye was proptosed and the cornea traumatized by making a 3-mm central corneal scratch into the anterior corneal stroma, using a sharp 25-gauge, 5/8-in bevel-up needle. One drop of an undiluted overnight broth culture of *P aeruginosa* was introduced onto the traumatized cornea. Each drop of suspension contained approximately 5×10^8 viable organisms.

Methods of Treatment.—For topical therapy, the 40-mg/ml solution of gentamicin sulfate was dilued with sterile water, and the pH was adjusted with filtered sodium hydroxide to yield two antibiotic solutions with concentrations of 20 mg/ml and 3 mg/ml.

Subconjunctival, periocular, and intramuscular injections were administered using 0.5 ml of the 40-mg/ml solution (20 mg gentamicin per injection).

The control solution was sterile saline (0.9%) used for both topical and subconjunctival administration.

All treatments began 24 hours after infection. The topical therapy regimen consisted of two drops of antibiotic (every 30 minutes) to each eye. Since previous experiments have shown no therapeutic carryover at these concentrations,[126] both eyes were used in topical trials.

Subconjunctival injections were given with 0.5 ml of the 40-mg/ml solution (20 mg gentamicin per injection) using a 25-gauge (5/8-in) needle on a tuberculin syringe to right eyes only. The needle was inserted subconjunctivally in the superotemporal quadrant 3 mm from the limbus, under Tenon's capsule, so at least one half of the needle shaft was under Tenon's capsule before the injection was given. Using this technique, no obvious leak was grossly seen around the suture tract, and a bleb was

raised. Using the same technique, four eyes of two rabbits received subconjunctival fluorescein (10%) to evaluate surface leakage.

Percutaneous-periocular injections were given with 0.5 ml of the 40-mg/ml solution (20 mg gentamicin) using a 25-gauge (⅝-in) needle through the skin of the lower lid.

Percutaneous-subconjunctival injections were made to place the solution subconjunctivally without a conjunctival needle tract, using 0.5 ml of the 40-mg/ml solution (20 mg gentamicin) with a 25-gauge (1½-in) needle. The needle was introduced through the shaved skin of the upper lid and threaded subconjunctivally to its final position near the limbus, leaving the conjunctival surface intact. The same technique was used in four eyes of two rabbits to administer fluorescein (10%) to evaluate surface leakage.

Intramuscular injections were given into the gluteal muscle. I administered or supervised the subconjunctival, percutaneous-periocular, and percutaneous-subconjunctival injections.

Quantitative Microbiology.—The following procedures were carried out:

1. *Method of assay*. The rabbits were killed with intraperitoneal sodium pentobarbital either 6 or 24 hours after beginning the treatment. Entire corneas were removed, washed with sterile saline, placed in 1.0 ml of Mueller-Hinton broth, and ground with an automatic pestle tissue homogenizer. Colony counts were determined by making serial dilutions in Mueller-Hinton broth and streaking 0.1-ml aliquots from each dilution onto trypticase soy agar plates. The plates were inverted and incubated overnight at 37 C. The number of colonies were counted by using a probe connected to an automatic counter.

2. *Antibiotic carryover*. An in vivo control study was done to assess possible topical antibiotic carryover immediately after treatment. Sixteen rabbit eyes were traumatized and infected, using the scratch method. Twenty-four hours after infection, half of the eyes were given a single treatment (two drops) of gentamicin solution (40 mg/ml) and compared with the other half receiving saline solution. The rabbits were killed immediately following treatment, the corneas were removed, rinsed with sterile saline solution, ground, and plated. After overnight incubation, the number of *Pseudomonas* colonies were counted.

An in vitro control study was done in 24 rabbits to assess possible antibiotic carryover after topical and subconjunctival administration of gentamicin. The corneas of all eyes of eight rabbits (topical group) and right eyes of 16 rabbits (subconjunctival group) were traumatized as previously described. In the topical group, one half of the eyes were given a single treatment (two drops) of gentamicin solution (40 mg/ml) and

the other half were given a topical saline solution. Eight rabbits of the subconjunctival group were given 0.5 ml of 40-mg/ml gentamicin subconjunctivally in the right eye only and the other eight rabbit eyes were given subconjunctival injections of 0.5 ml of sterile saline solution. Immediately after topical treatment and six hours after subconjunctival treatment, the rabbits were killed, the corneas were removed, rinsed with sterile saline solution, and put into tissue grinder tubes along with 0.9 ml of Mueller-Hinton broth and 0.1 ml of 10^{-4} dilution of an overnight broth culture of *P aeruginosa*. Each tube contained approximately 9.3×10^4 *Pseudomonas* organisms per milliliter. The corneas were ground and plated. Following overnight incubation, the total count per milliliter was determined, using the appropriate dilution factor.

Statistics.—Numbers of viable bacteria in the corneas are presented as arithmetic means (equivalent to geometric mean) and SDs of common logarithms, base 10. This method of analysis mitigates the effect of single extreme values and is commonly used in analysis of numbers of microorganisms. Statistical analysis of the results was done by one-way analysis of variance and by the method of least significant differences.[127]

RESULTS

SEQUENTIAL ELECTRON MICROSCOPY STUDIES

CLINICAL EVALUATION

Clinical biomicroscopic evaluations of *Pseudomonas* keratitis, induced by superficial corneal trauma and topical inoculation of viable *P aeruginosa* organisms, were made at 8, 16, 24, 48, 72, and 96 hours. Control traumatized corneas showed mild stromal edema immediately surrounding the scratch wound, which did not progress to infiltration, and disappeared within 24 to 48 hours after wound epithelialization. A reaction of mild anterior chamber inflammation was usually noted only within the first 24 hours. In the infected eyes, early conjunctival reaction with bulbar conjunctival hyperemia, limbal hyperemia, and a mild purulent conjunctival discharge were usually noted at 8 hours, became moderate at 24 hours, and extremely severe after 48 hours.

In the infected eyes, moderate corneal stromal edema and multiple dense focal punctate or irregular linear superficial infiltrates were noted surrounding the linear ulcerated wound at eight hours (Fig 4A). The corneal edema and infiltration surrounding the ulcerated wound frequently became an inflammatory halo within 16 hours, and a confluent oval area of edema and infiltrate 24 hours after infection (Fig 4B). Forty-eight hours after infection, the infiltrate became more dense centrally and

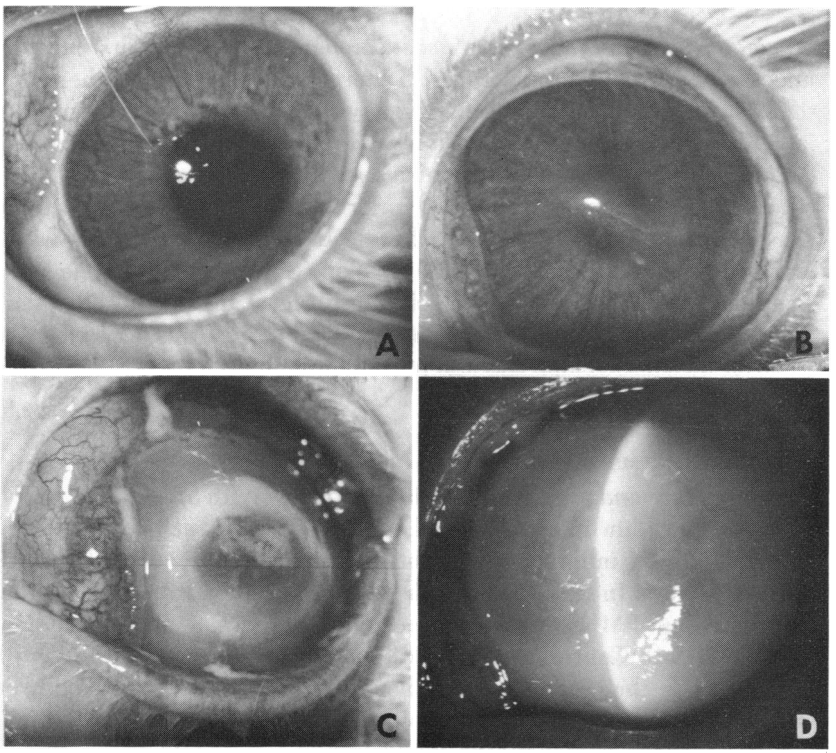

FIGURE 4
A: *Pseudomonas* keratitis in rabbit eight hours after infection. B: *Pseudomonas* keratitis in rabbit 24 hours after infection. C: *Pseudomonas* keratitis in rabbit 48 hours after infection. D: *Pseudomonas* keratitis in rabbit 96 hours after infection.

enlarged peripherally to include most of the corneal diameter. Peripheral, incomplete, ring-shaped infiltrates were seen in most eyes (Fig 4C), and the limbal cornea was uniformly edematous and infiltrated at 48 hours. Observations at 72 to 96 hours showed progressive, diffuse, dense, white infiltration and marked stromal edema from limbus to limbus, with areas of marked anterior stromal breakdown and thinning in some corneas (Fig 4D).

All of the anterior chambers in the infected eyes had a mild inflammatory reaction at 8 hours, a moderately severe inflammatory reaction at 24 hours (occasionally with hypopyon), and an extremely severe anterior chamber reaction with a consistent hypopyon at 48 hours. Anterior chamber details were usually obscured by corneal opacity within 72 to 96 hours.

SEM, TEM, AND LIGHT MICROSCOPY

Early adhesion and invasion of Pseudomonas.—Within the first hours of infection, SEM demonstrated that bacteria were selectively adhering to the area of the wound, but only rarely were bacteria found adhering to intact or uninjured epithelial cells away from the wound (Figs 5 through 8). At 15 minutes, bacteria were seen with TEM adjacent and selectively adhering to the stromal wound edge prior to stromal invasion by bacteria. The organisms were surrounded by electron-lucent envelopes, which may represent a glycocalyx or slime envelope (Fig 9 and 10). At one hour, some bacteria were adhering to collagen fibrils at the wound edge, and definite penetration and invasion into corneal stroma was occurring. An electron-lucent envelope was still present around each bacterium (Fig 11 and 12).

MECHANISMS IN PMN RESPONSE

Early response and source of PMN infiltration.—With SEM, a few PMNs were noted within infected wounds of some corneas at two hours, but significant PMN infiltration was not prominent in the infected corneas until four hours. In some corneas, the wound was filled with PMNs within four hours, but PMNs were never seen on the surface of intact epithelium (Fig 13 and 14). Control traumatized, noninfected corneas showed only a few PMNs in the wounds at four hours, and they usually disappeared within 48 hours after the wound epithelialized.

Light sections demonstrated infiltration of PMNs into the wound at eight hours (Fig 15) and progression of infiltration around the wound with progressive epithelial breakdown at 16 hours (Fig 16). At 24 hours, the PMN infiltration extended into the midstroma, and inflammatory cells were seen on the endothelium and in the anterior chamber. The PMN infiltration appeared to originate from the ulcerated area and spread deeper into the stroma and centrifugally from the ulcer through the stroma toward the limbus (Fig 17). Only mild PMN infiltration was seen in the peripheral cornea at 24 hours, and the limbal-to-central ulcer PMN movement was insignificant. At approximately 48 hours, a dense infiltration of PMNs was seen surrounding limbal vessels and in peripheral corneal stroma (Fig 18).

Early PMN Phagocytosis and Digestion.—With TEM, an occasional PMN was already seen in the anterior corneal stroma at two hours after infection (Fig 19), but PMN infiltration into the anterior corneal stroma was not significant until four hours after infection. Phagocytosis and digestion of bacteria by PMNs was already noted in the wound four hours after

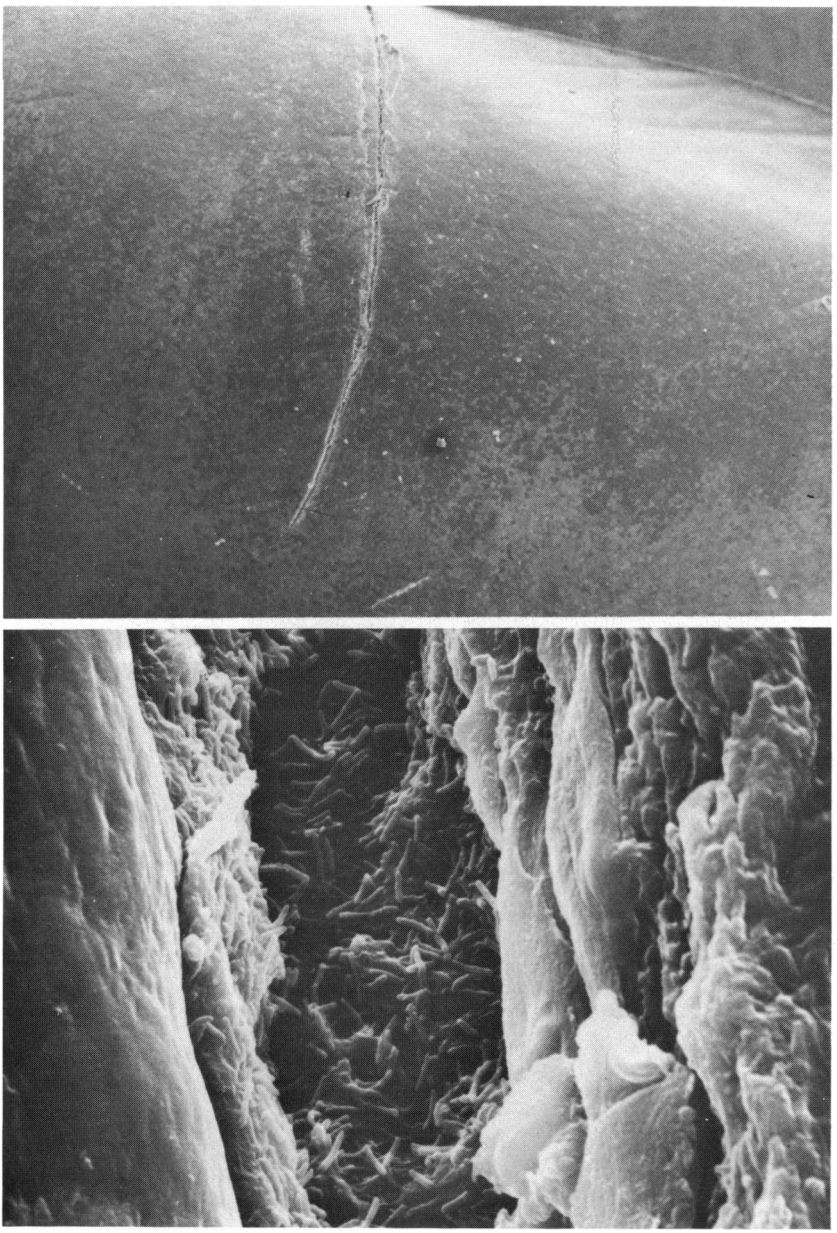

FIGURE 5

Top: SEM of corneal wound one hour after scratch injury and infection. Light and dark superficial epithelial cells showing normal patchwork quilt appearance to intact epithelium on either side of scratch (×20). Bottom: Multiple rod-shaped bacteria can be seen in bottom and along sides of scratch at high magnification (×2,000).

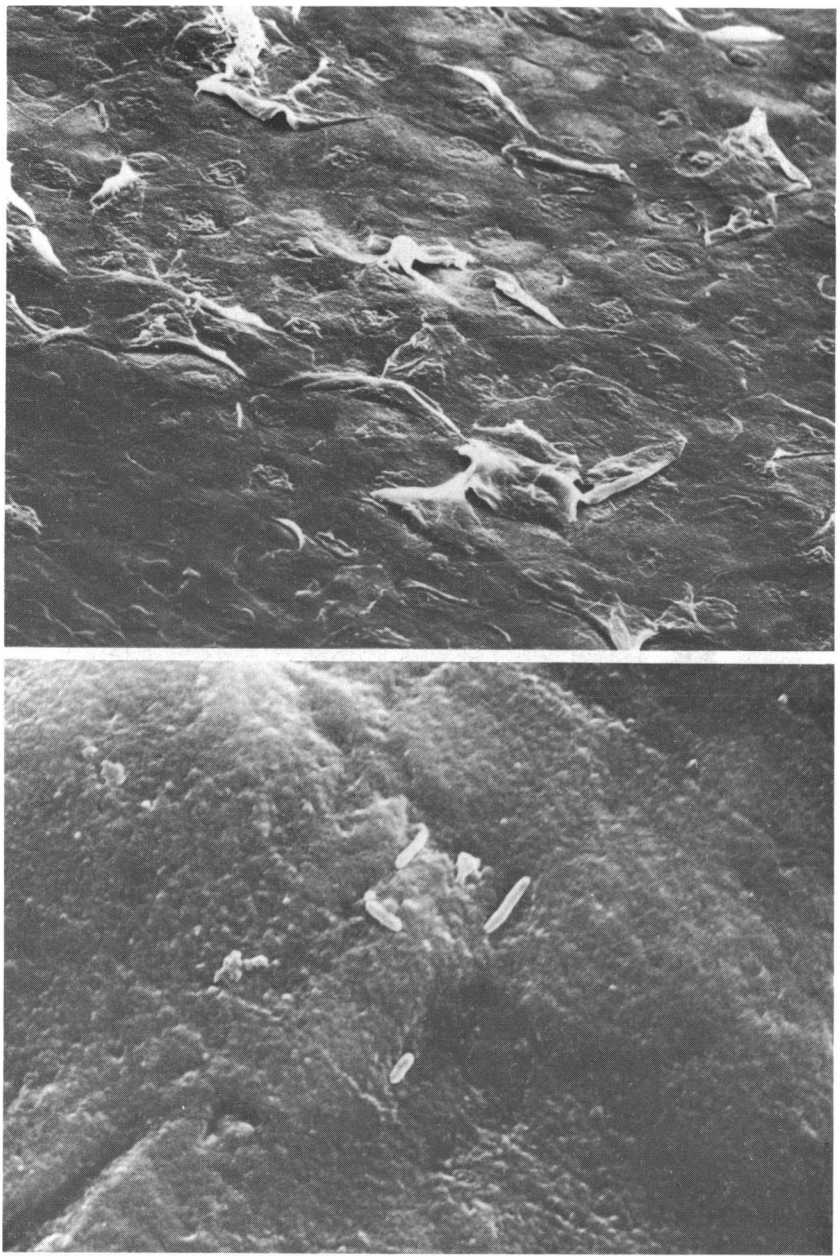

FIGURE 6
SEM of superficial epithelium of same cornea as in Fig 5 at low magnification (×500) (top) and high magnification (×2,200) (bottom). Only few rod-shaped bacteria could be found adhering to uninjured or intact epithelial cells.

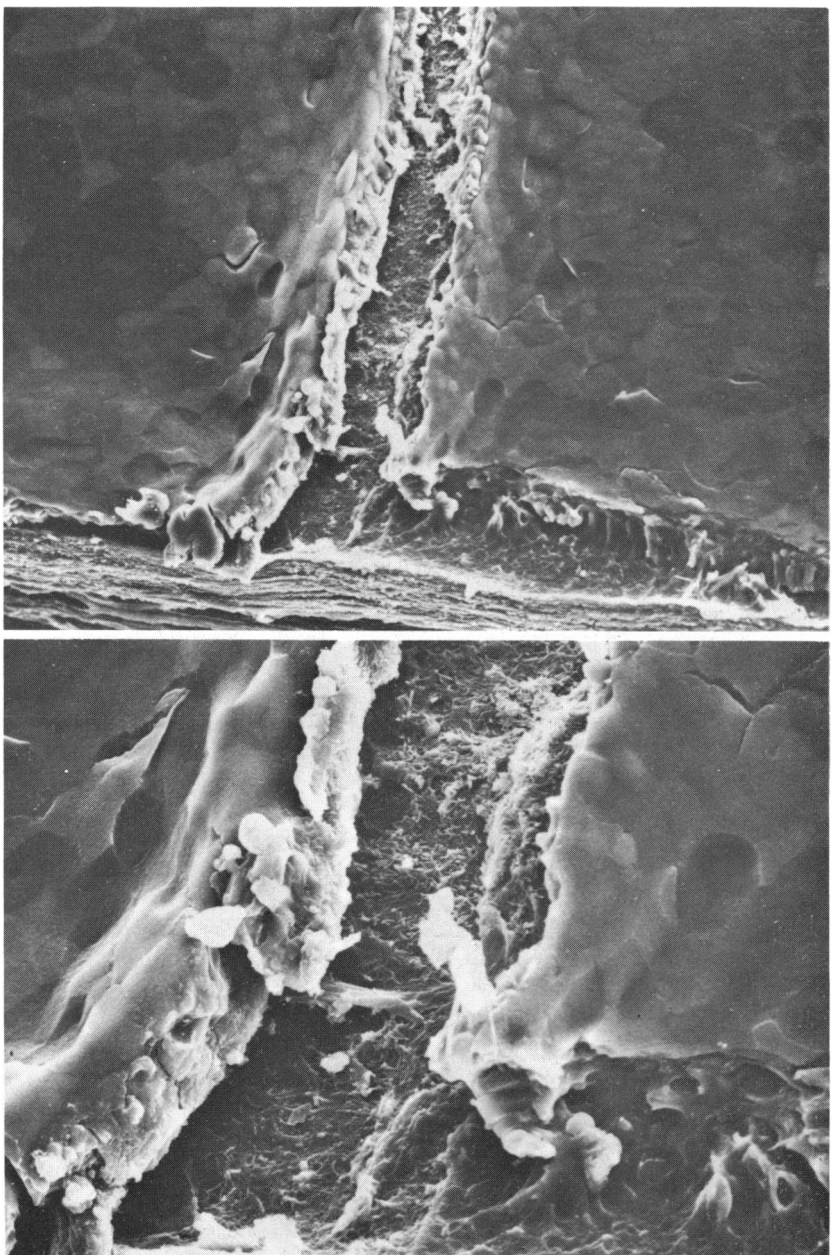

FIGURE 7
SEM of wound two hours after scratching at low magnification (×200) at top and medium magnification (×500) at bottom. No PMNs or bacteria were seen on epithelial surface.

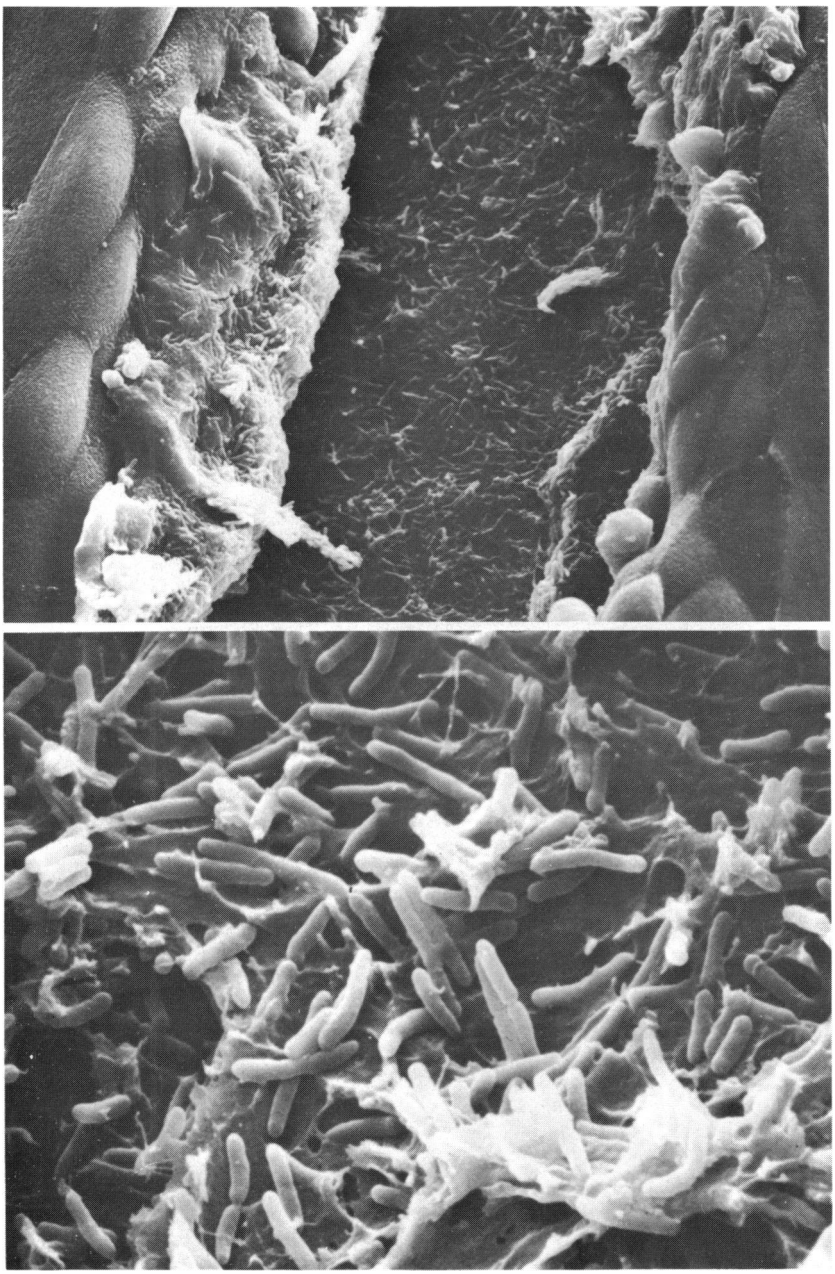

FIGURE 8
Higher magnifications of same wound. Multiple rod-shaped bacteria were seen in base and on sides of wound. Magnification is ×1,000 at top and ×5,000 at bottom.

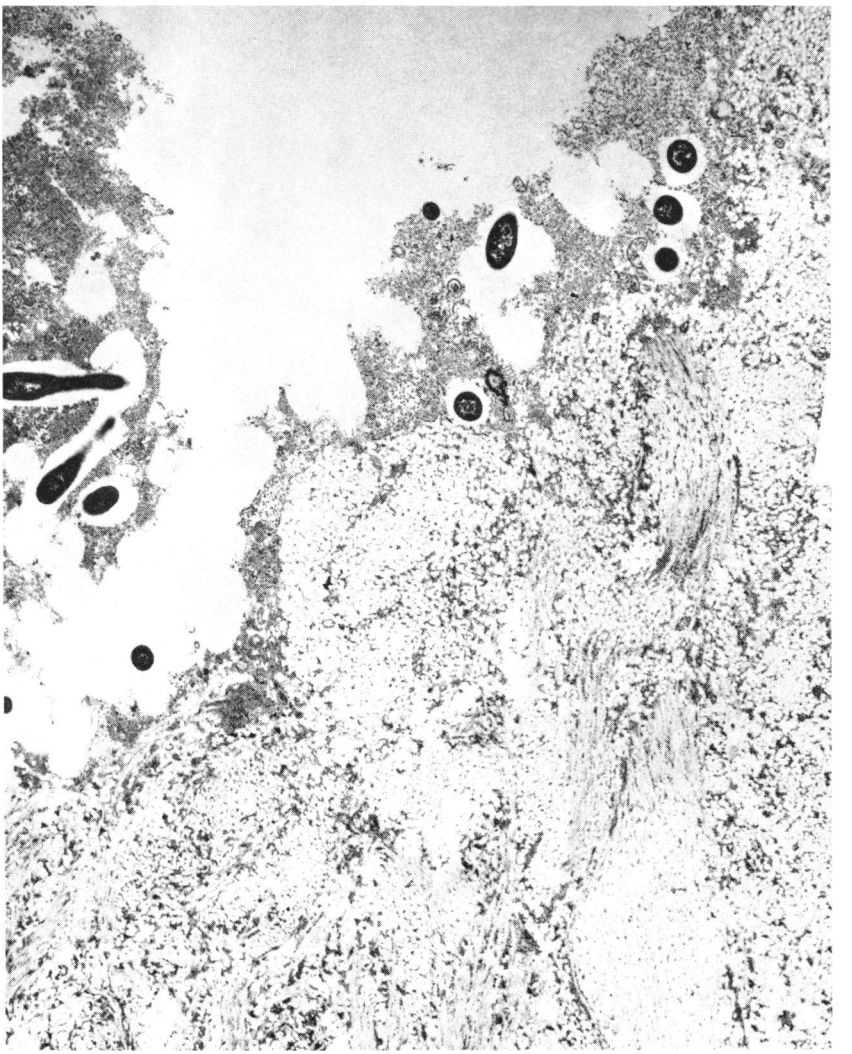

FIGURE 9
TEM of wound 15 minutes after infection. Bacteria are adjacent to wound edge deep in wound before stromal invasion (×8,600).

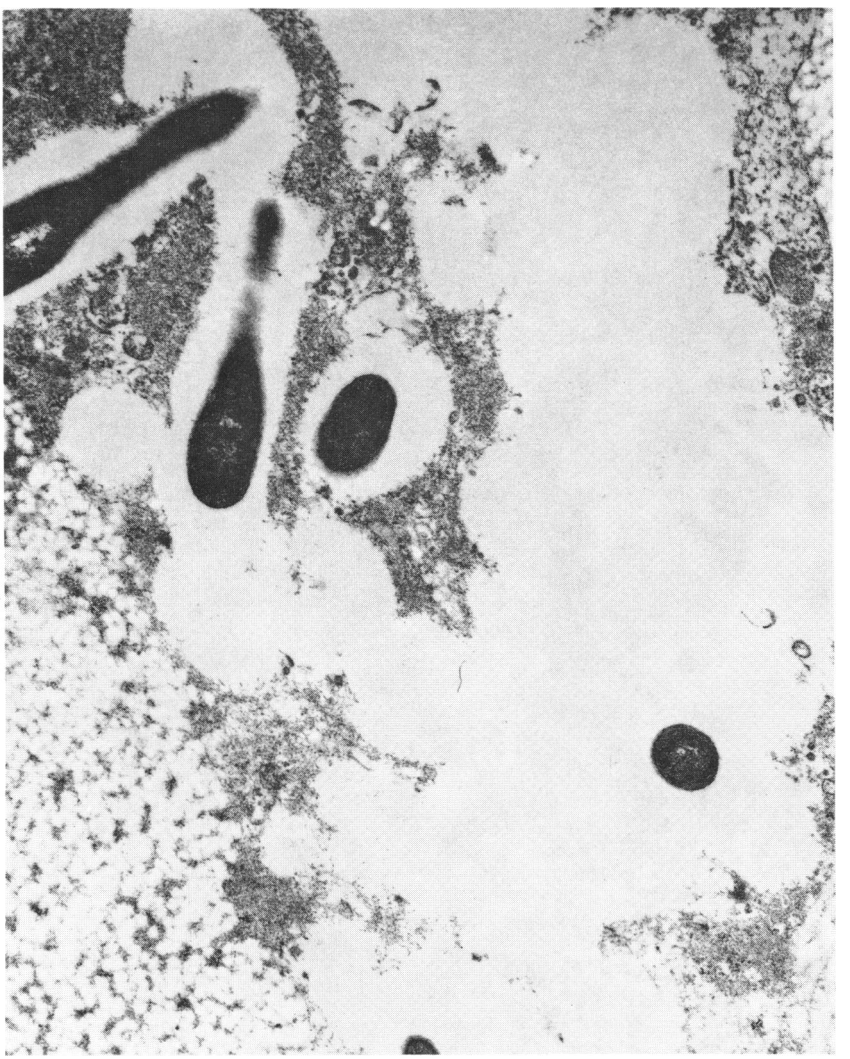

FIGURE 10
Higher magnification (×22,800) of TEM shown in Fig 9. Note empty, electron-lucent envelope around each bacterium.

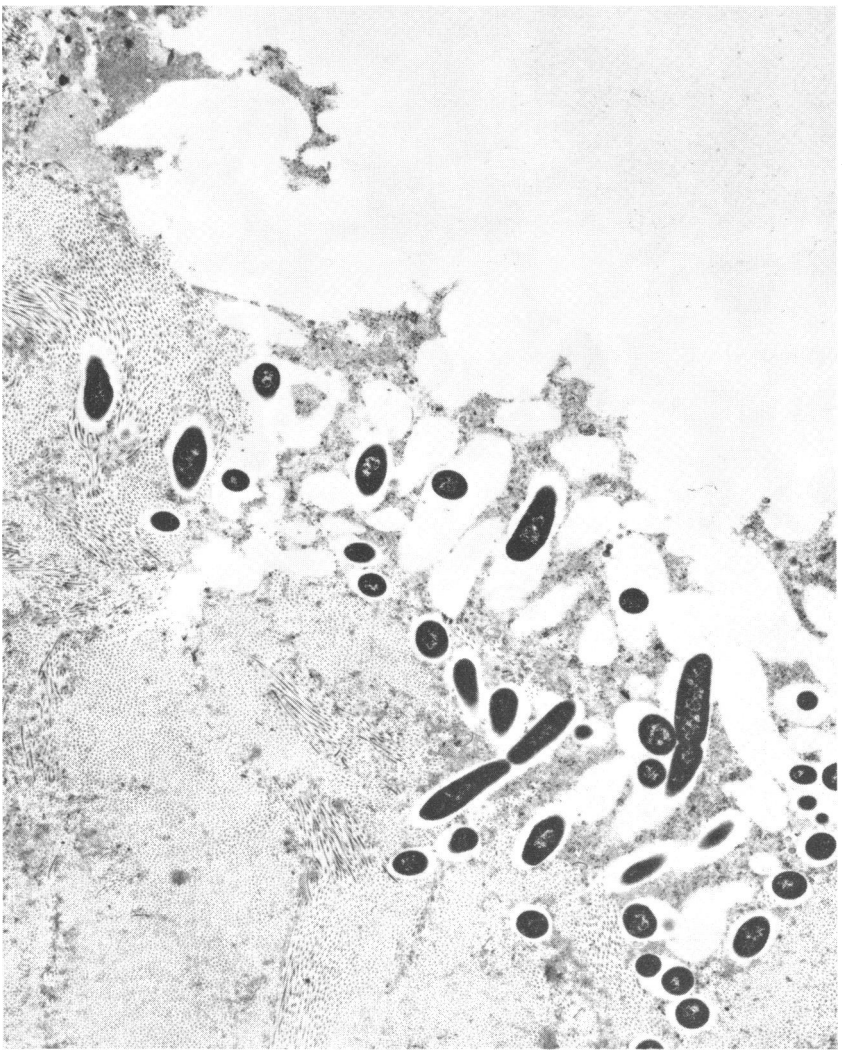

FIGURE 11
Adherence and initial invasion of bacteria into stromal edge of wound one hour after infection. Note electron-lucent envelope around each bacterium (×8,600).

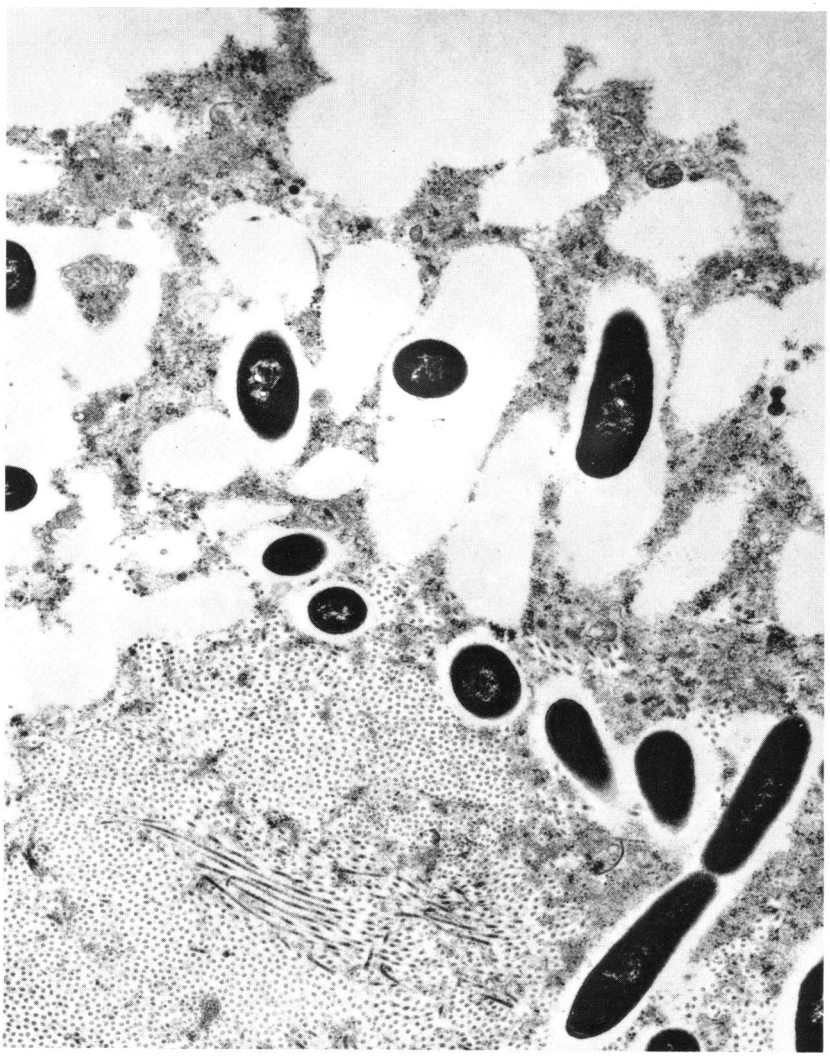

FIGURE 12
Higher magnification of Fig 11 (× 16,600).

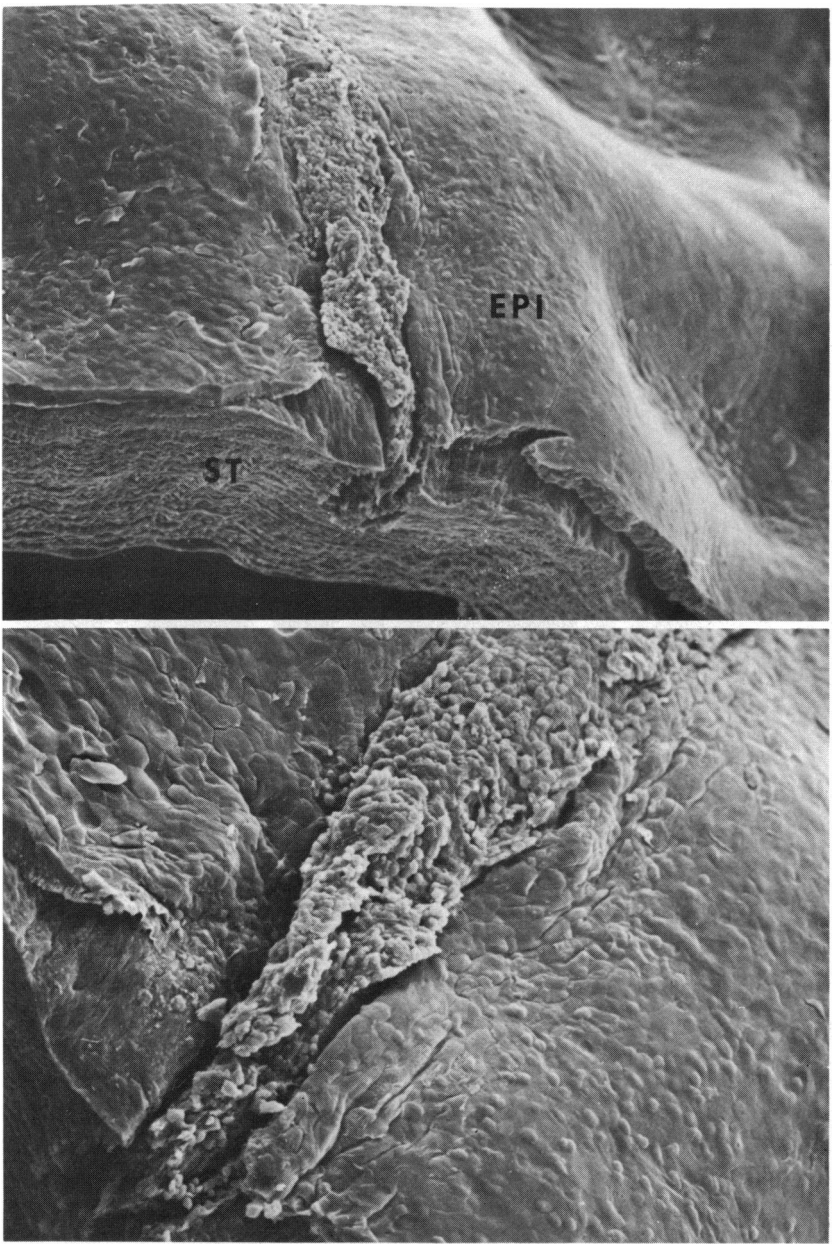

FIGURE 13
SEM of wound in corneal epithelium (EPI) and stroma (ST) four hours after infection. Wound was filled with PMNs but none were seen on surface of intact epithelium (×100 at top and ×200 at bottom).

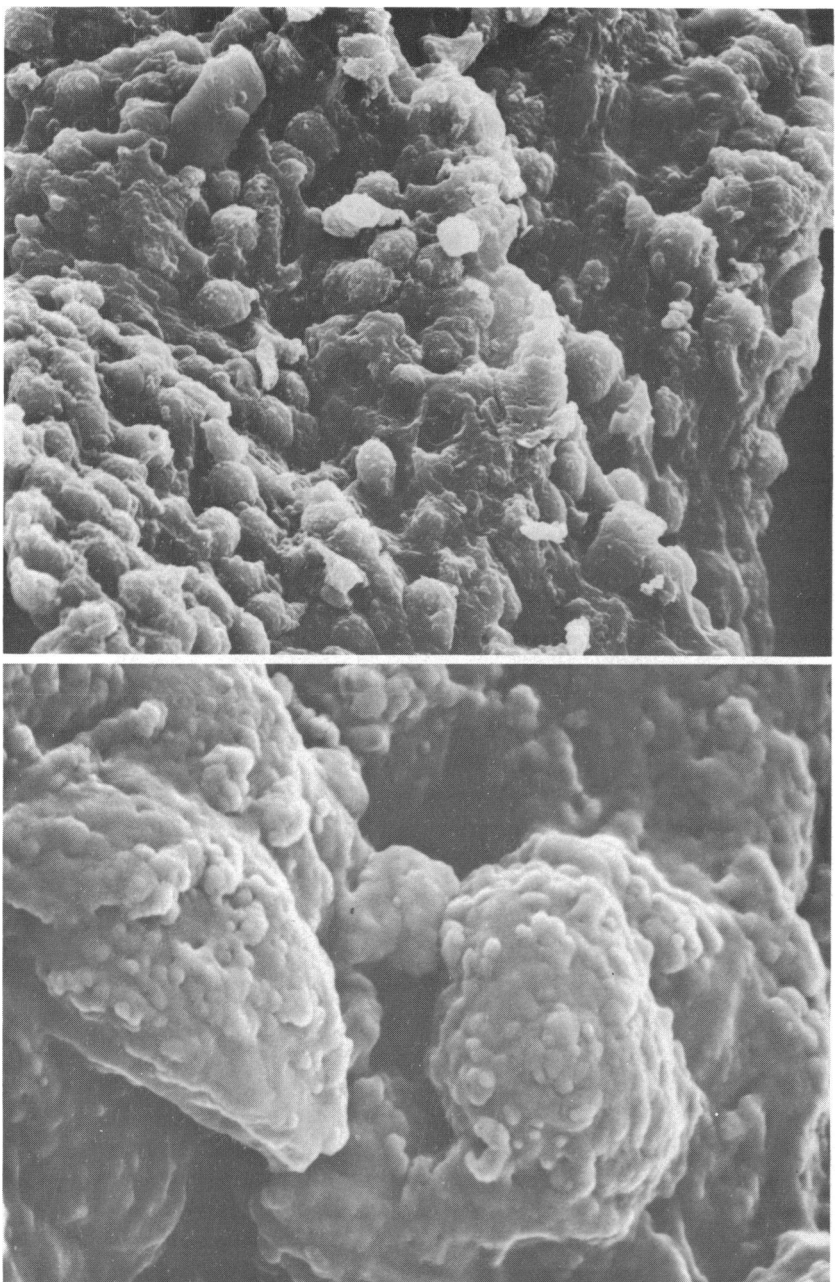

FIGURE 14
Higher magnification of PMNs in wound (×1,000 at top and ×5,000 at bottom).

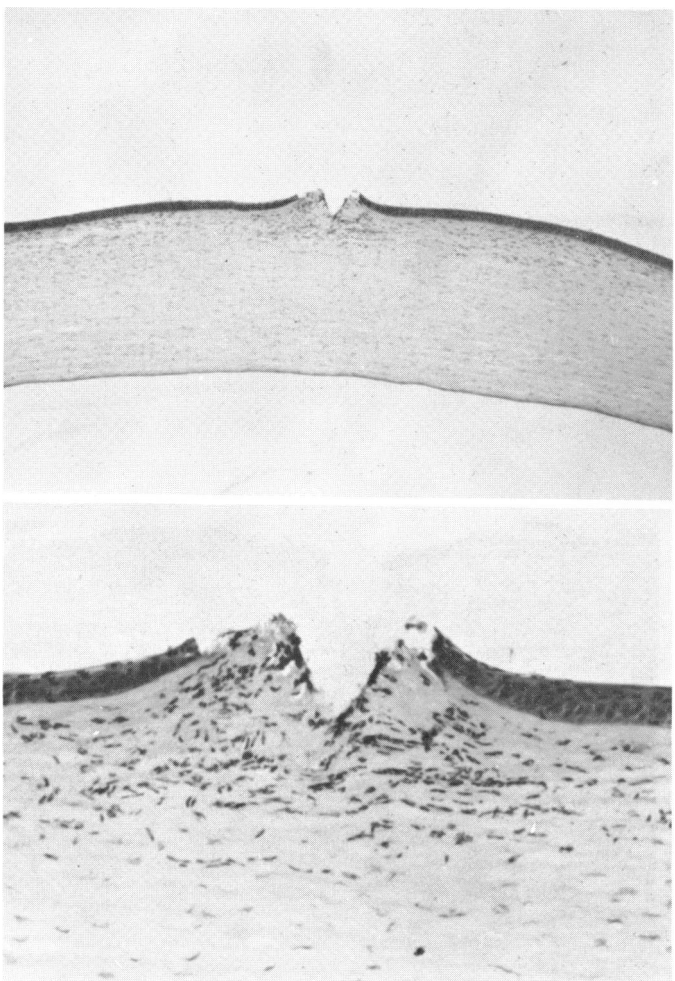

FIGURE 15
Light micrograph of cross-section of cornea eight hours after infection. PMNs are seen infiltrating stroma through wounded area (×16 at top and ×63 at bottom).

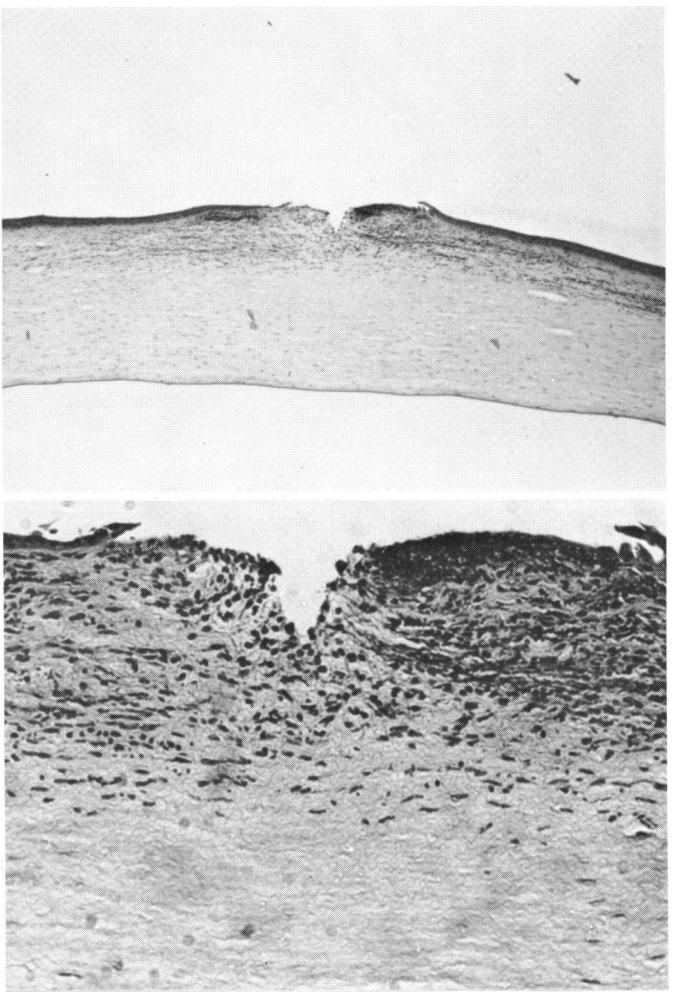

FIGURE 16
Light microscopy of cross-section of cornea 16 hours after infection. PMNs have infiltrated anterior stroma and adjacent epithelium is breaking down (× 16 at top and × 63 at bottom).

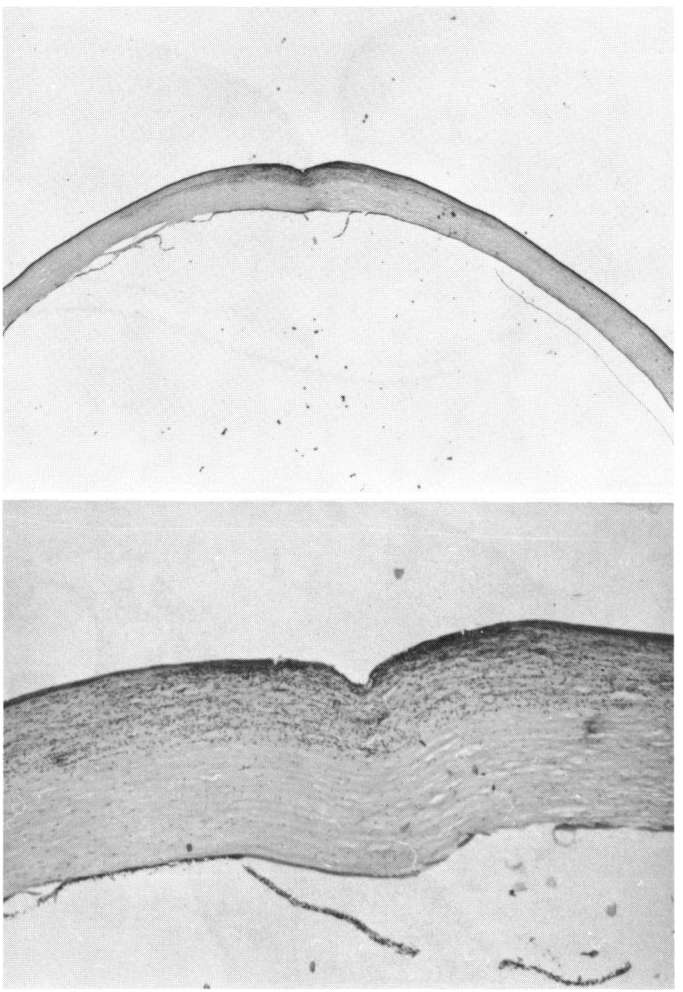

FIGURE 17

Light micrographs of cornea 24 hours after infection. PMN infiltrate has extended into midstroma, and inflammatory cells were present in anterior chamber. PMNs have entered from ulcerated area and have spread centrifugally. At this stage, only mild PMN infiltration was apparent in peripheral cornea (×8 at top and ×16 at bottom).

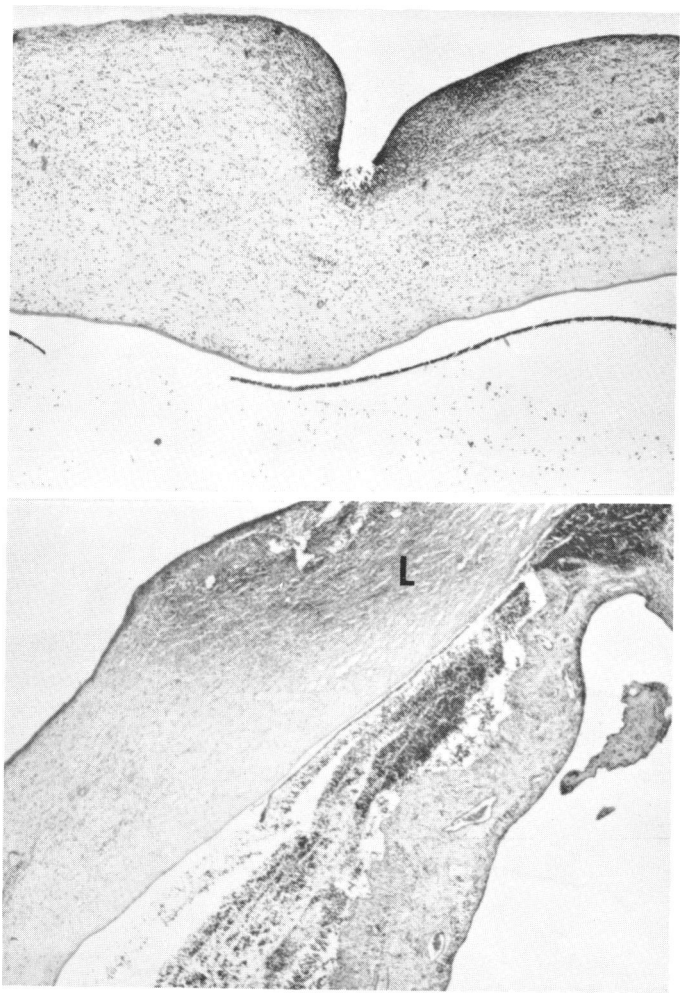

FIGURE 18
Light micrographs of cornea 48 hours after infection. Top, PMNs have extended into deep stroma centrally (×16). Bottom, Dense PMN infiltration of limbus (L) and peripheral corneal stroma (×12).

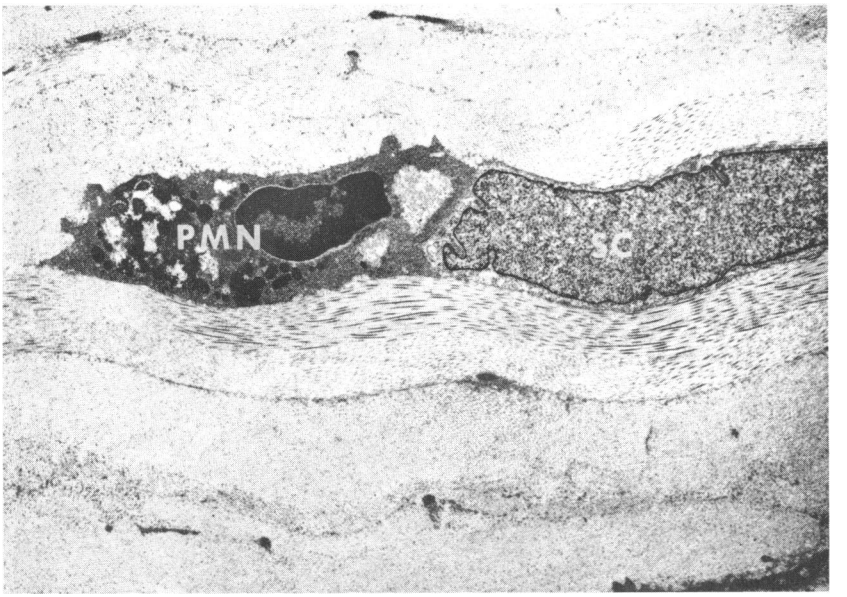

FIGURE 19
Intact PMN migrating into anterior stroma of cornea two hours after infection. Adjacent stromal cell (SC) appears normal (×8,600).

infection (Fig 20 and 21) and in the anterior corneal stroma eight hours after infection (Fig 22).

Epithelial cell loss in ulceration.—PMN infiltration was seen under the corneal epithelium adjacent to the ulcer, associated with an absence of most of the epithelial basement membrane immediately over the degradating PMNs (Fig 23). The epithelium adjacent to the ulceration no longer has basement membrane and has permitted PMNs to enter the intracellular space (Fig 24). This may be the mechanism for the progression in epithelial loss seen previously (Fig 16).

Interactions and damage in corneal stroma.—Stromal keratocytes became necrotic and were often already digested at four and eight hours after infection. They were sensitive to the presence of bacteria or PMNs in their area, and were the first structures to degenerate in an area of early inflammation.

Localized areas of probable early collagen degradation with electron-dense particles were apparent in certain areas around degranulated PMNs 8 and 16 hours after infection (Fig 22 and 23). However, significant evidence of collagen damage was not seen until 24 hours after infection.

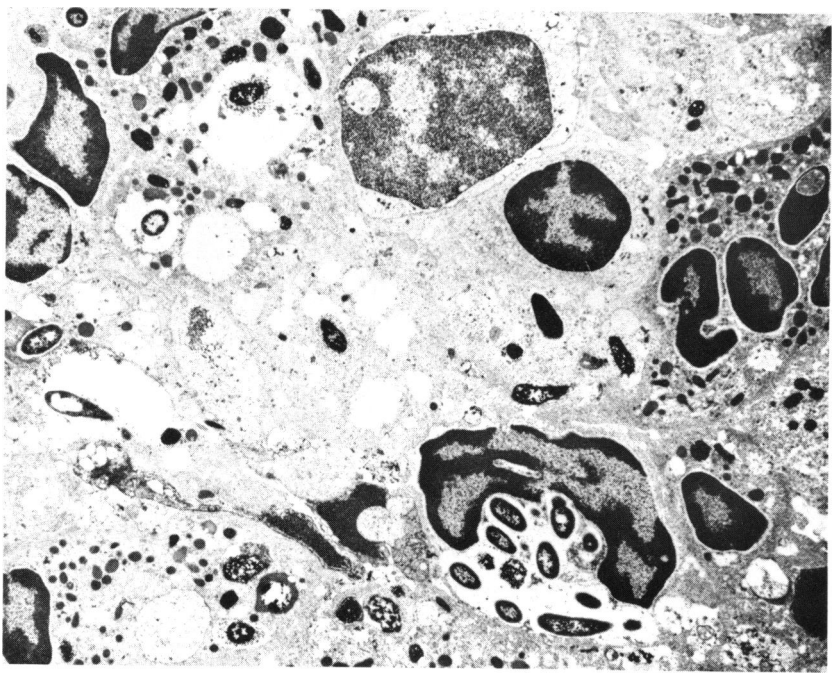

FIGURE 20
Aggregate of PMNs in wound four hours after infection containing phagocytized bacteria in various stages of digestion (×6,400).

In many areas of anterior corneal stroma, extensive collagen degradation was noted at 24 hours with loss of collagen fibrils, and accumulation of electron-dense granules in association with degranulated PMNs (Fig 25 and 26). In the periphery of the ulcer at 24 hours, bacteria were seen on the corneal surface both free in corneal stroma and phagocytized by PMNs (Fig 27 and 28). Most of the bacteria seen at 24 hours demonstrated no, or only a slight, electron-lucent envelope (Fig 28 and 29), compared with the envelope seen in the earlier hours of infection.

In the periphery of the ulceration, PMNs were still intact and penetrating anterior stroma from the corneal surface at 48 hours (Fig 30). Ninety-six hours after infection there was extensive collagen degradation and cellular debris in the anterior corneal stroma (Fig 31). In some areas, intact PMNs were seen on the corneal surface and were apparently removing necrotic debris (Fig 32). In midcorneal stroma at 96 hours, there was extensive loss of collagen fibers, widespread accumulation of electron-dense particles, electron-dense amorphous material, and prominent tactoid formation in many areas (Fig 33).

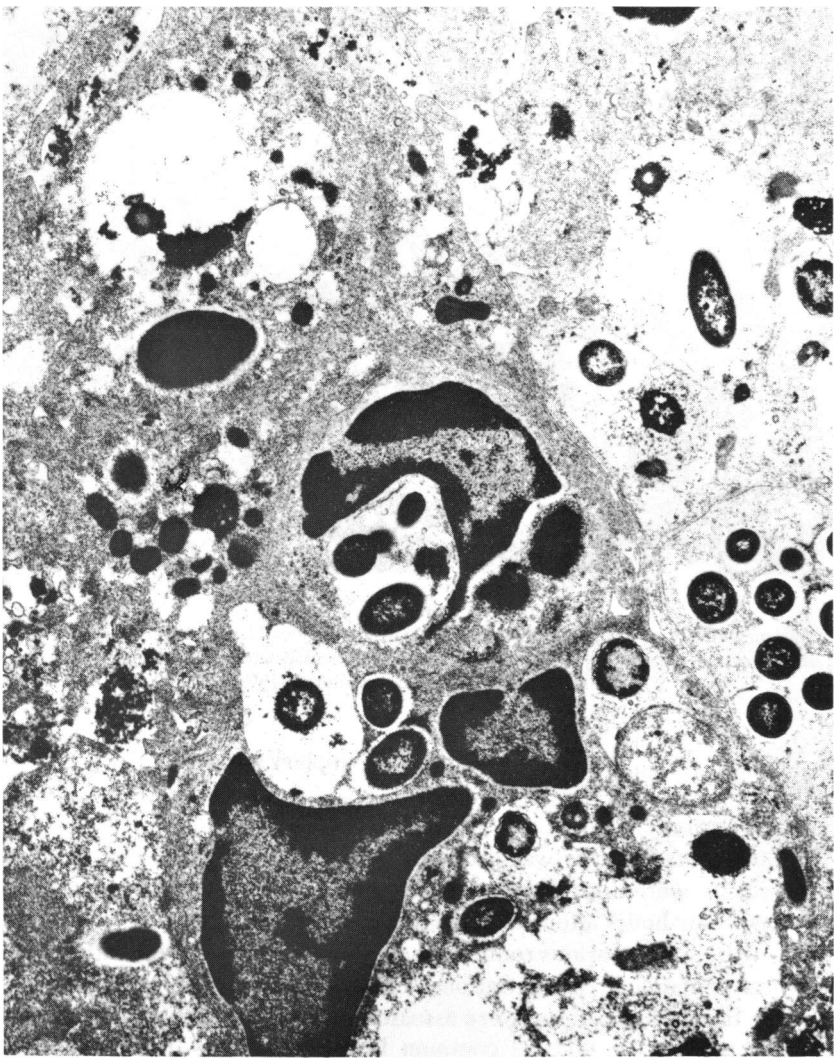

FIGURE 21
Higher magnification of phagocytized bacteria (× 12,000).

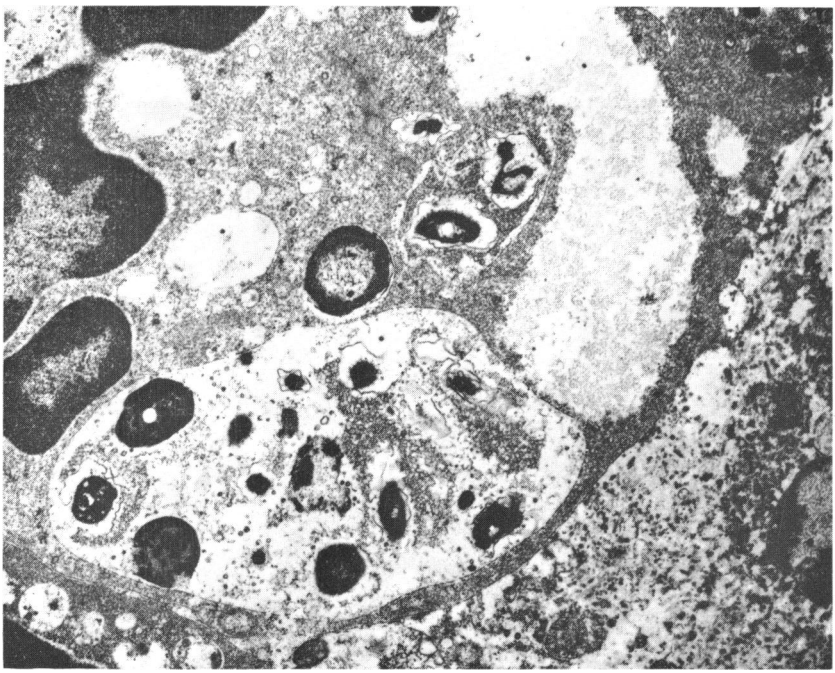

FIGURE 22
High magnification of phagosome containing partially digested bacteria in PMN eight hours after infection. Probable early collagen degradation is associated with electron-dense particles in lower right-hand corner (× 16,600).

EXPERIMENTAL KERATITIS/THERAPY STUDIES

INTRODUCTION

Pseudomonas keratitis was produced in rabbits using a model in which organisms were introduced topically after superficial corneal trauma. Twenty-four hours after infection the keratitis was treated with gentamicin sulfate, using various routes and commercially equivalent and fortified topical gentamicin concentrations. Following treatment, the number of viable bacteria remaining were assessed using quantitative microbiologic assays reported as SDs of common logarithms, base 10, for statistical analysis of the data. Control studies assessed possible antibiotic carryover after topical and subconjunctival treatment. No antibiotic carryover occurred with either topical treatment, even when higher concentrations of antibiotic solutions were evaluated, or with subconjunctival treatment (Table II and III).

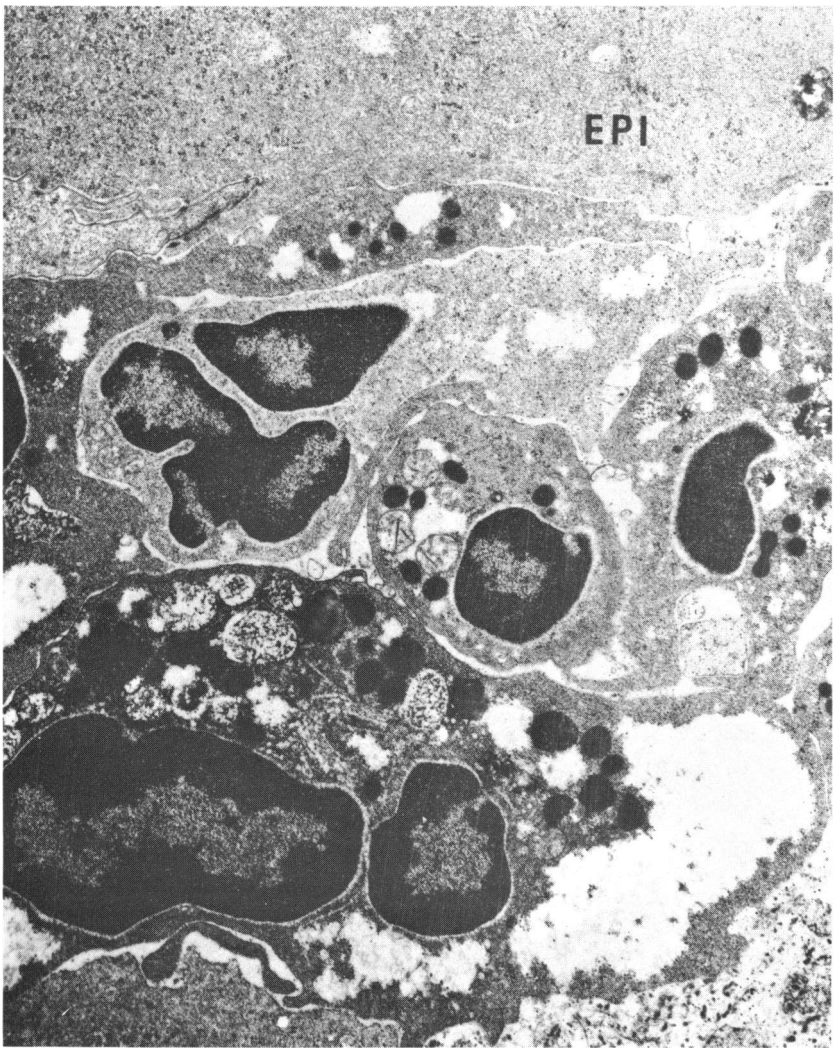

FIGURE 23
PMNs beneath corneal epithelium (EPI) near ulcer 16 hours after infection. Note interruption of epithelial basement membrane in micrograph. Early collagen degradation was seen around PMNs at lower right (× 12,000).

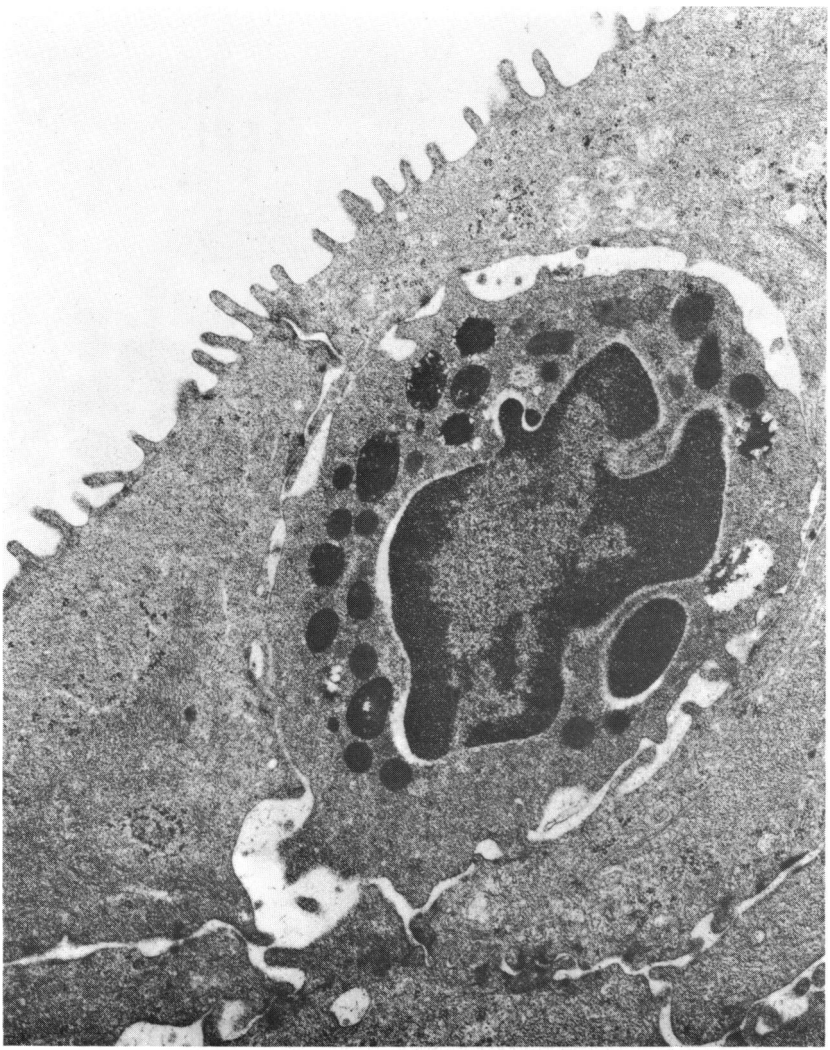

FIGURE 24
PMN between intact superficial epithelial cells in same cornea as in Fig 23 (× 16,600).

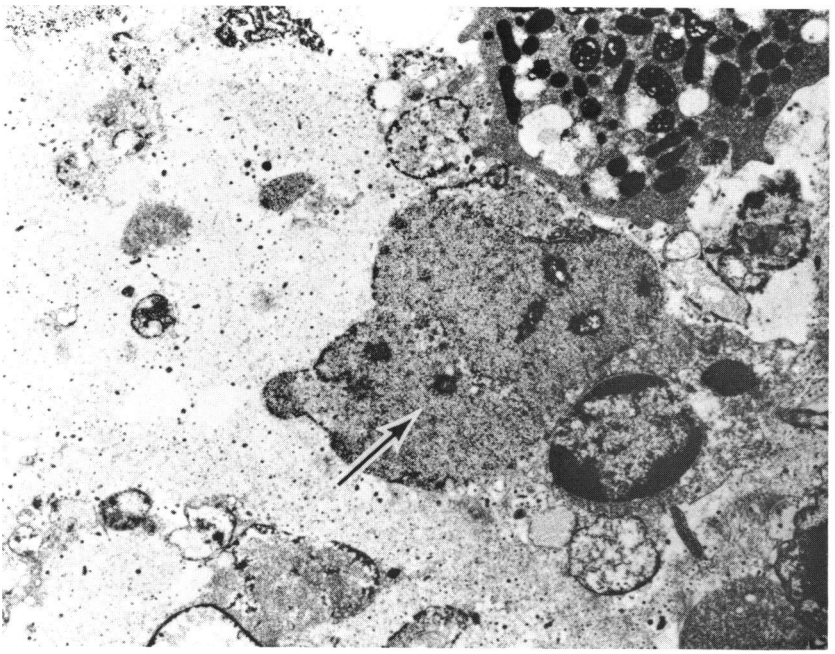

FIGURE 25
Extensive collagen degradation with loss of collagen fibrils and accumulation of electron-dense granules seen in association with degranulated PMNs (arrow) 24 hours after infection (×12,000).

TOPICAL VERSUS SUBCONJUNCTIVAL SIX-HOUR THERAPY TRIALS

The efficacy of topical gentamicin (20 mg/ml) alone and subconjunctival gentamicin alone was determined. The topical fortified gentamicin (20 mg/ml) groups and the subconjunctival gentamicin (20 mg) groups all showed a good therapeutic effect in two separate six-hour therapy trials using two different strains of *P aeruginosa* (Table IV and V). No significant difference was noted between the topical and subconjunctival therapy groups in either six-hour trial. A mild but statistically significant ($P = .003$) effect was seen in the contralateral eyes of the subconjunctival group in one therapy trial (Table IV), which may have represented some systemic antibiotic carryover. This effect was not seen in the untreated eyes of the other trial (Table V) or in subsequent experiments. However, it reinforced the need to use only one eye when evaluating subconjunctival therapy because of possible, although infrequent, cross-over effect, possibly because of serum antibiotic concentrations that can be achieved in rabbits after administration of subconjunctival antibiotics.[110]

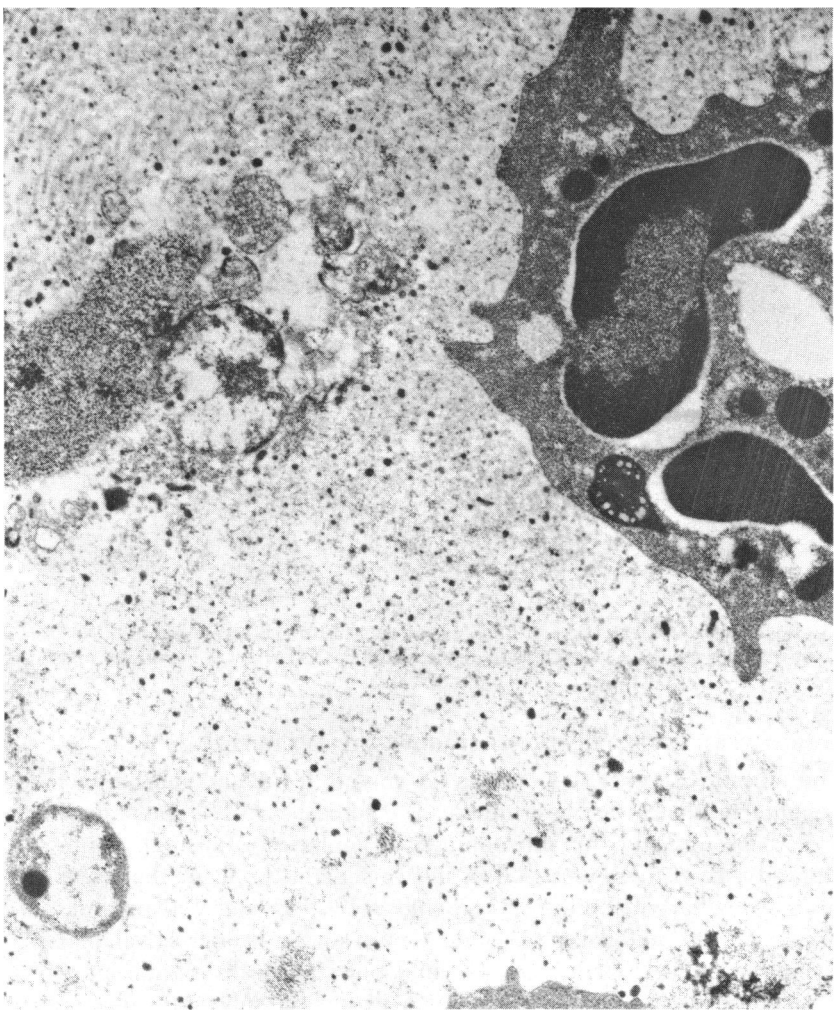

FIGURE 26
Cellular debris, lack of collagen fibrils, and accumulation of electron-dense granules as seen at higher magnification in same cornea shown in Fig 25 (×16,600).

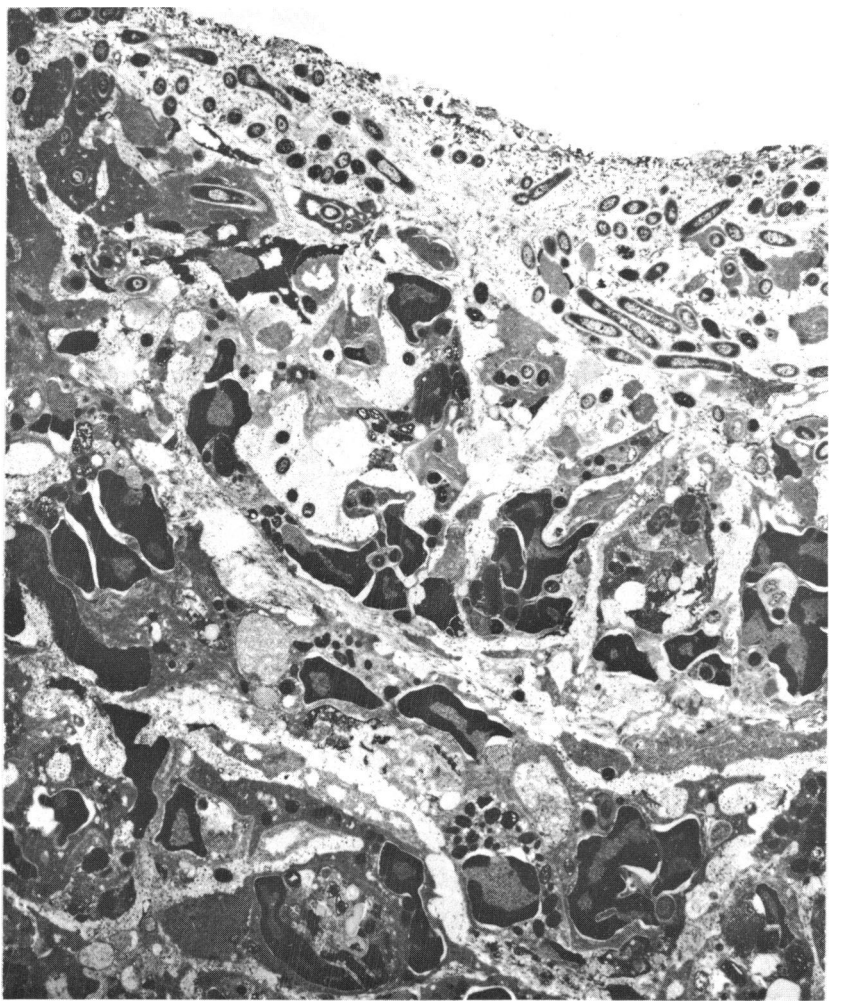

FIGURE 27

Corneal surface near periphery of ulcer, away from original wound, 24 hours after infection. Note absence of epithelium and basement membrane and accumulation of bacteria, both free in stroma and phagocytized by PMNs. Most of electron-lucent envelope surrounding most bacteria is absent, as compared with earlier stages of infection (×4,600).

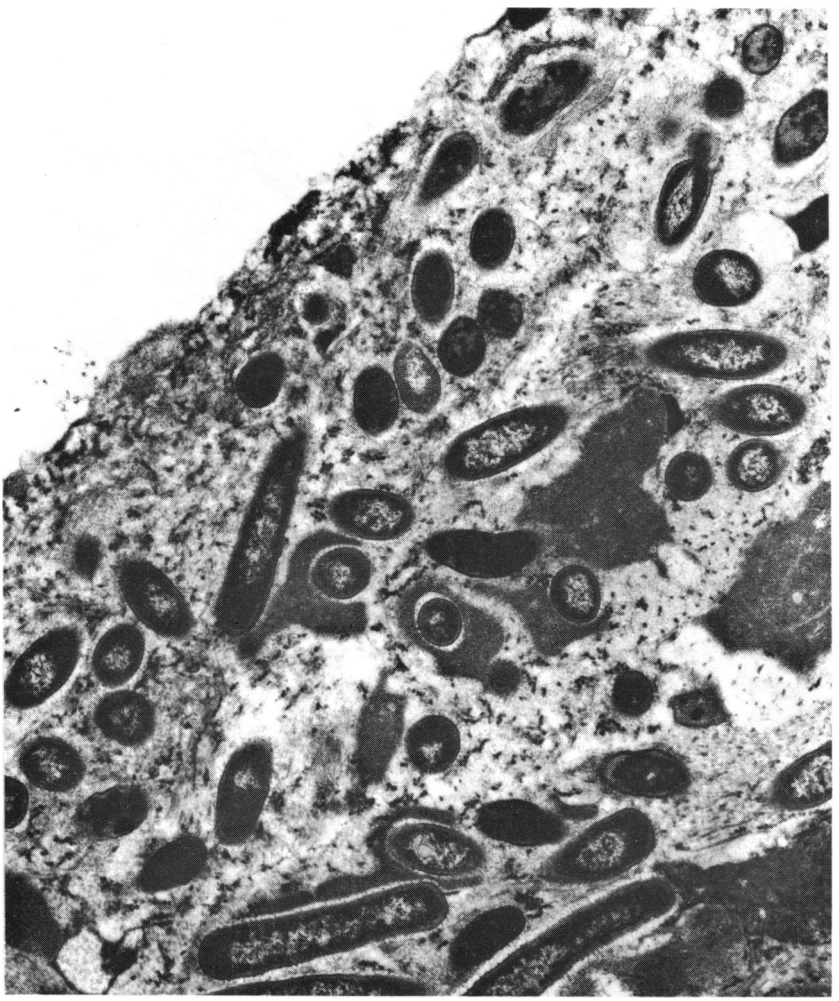

FIGURE 28
Higher magnification of bacteria in anterior stroma of same cornea shown in Fig 27 (×16,600).

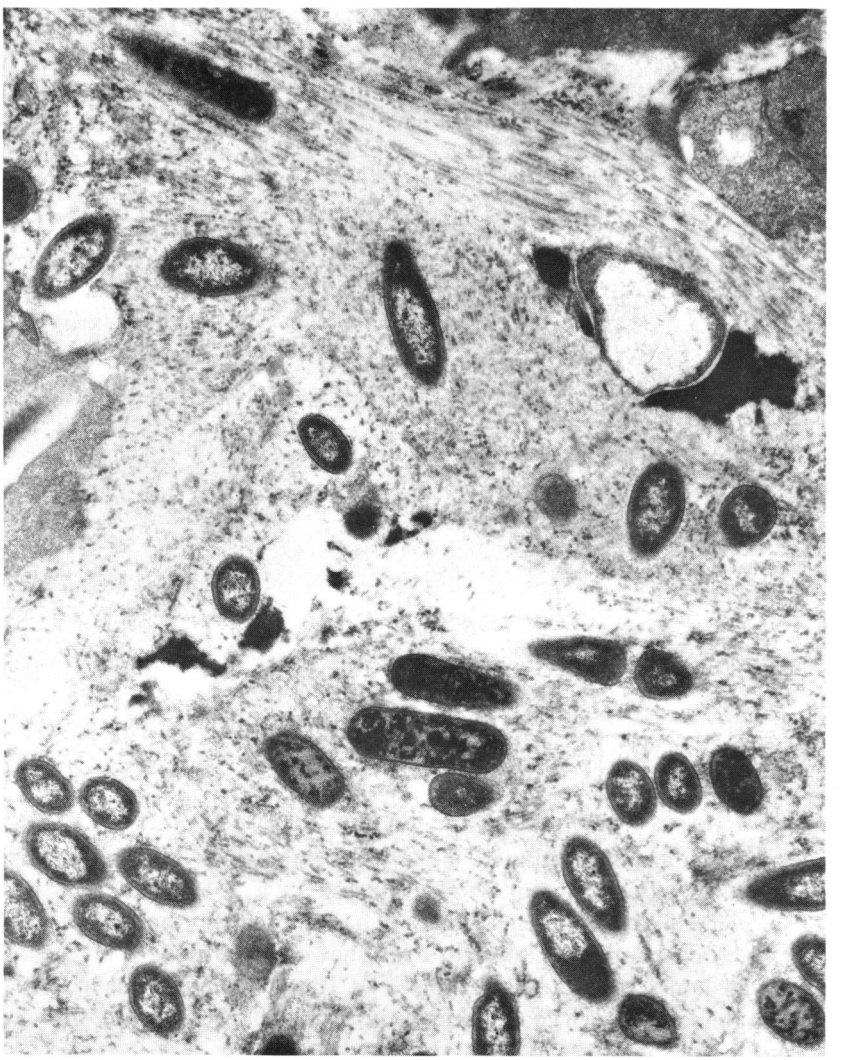

FIGURE 29
Bacteria deeper in stroma peripheral to original wound in same cornea shown in Fig 27. Note loss of most of electron-lucent envelope (× 16,600).

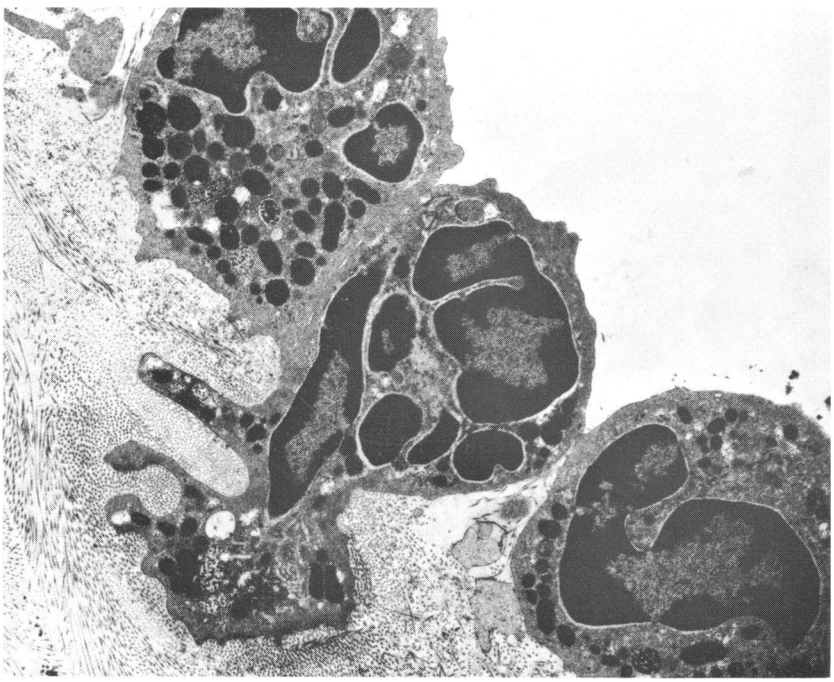

FIGURE 30
PMN penetrating anterior surface of ulcerated cornea midway between original wound and limbus 48 hours after infection (×12,000).

TOPICAL VERSUS SUBCONJUNCTIVAL 24-HOUR THERAPY TRIAL

Topical gentamicin (20 mg/ml) alone and a single dose of subconjunctival gentamicin alone (20 mg) were evaluated in a 24-hour therapy trial. Topical fortified gentamicin (20 mg/ml) had an excellent therapeutic effect in the group, and eight of eight eyes showed no growth of organism(s) after 24 hours of treatment (Table VI). The subconjunctival treatment group demonstrated a good therapeutic result, but it was significantly less than that of the topical antibiotic group.

TOPICAL VERSUS TOPICAL-SUBCONJUNCTIVAL (FORTIFIED CONCENTRATION OF GENTAMICIN TOPICALLY) THERAPY TRIAL

The next six experiments were designed to determine whether antibiotic given by the subconjunctival injection route would improve the effective-

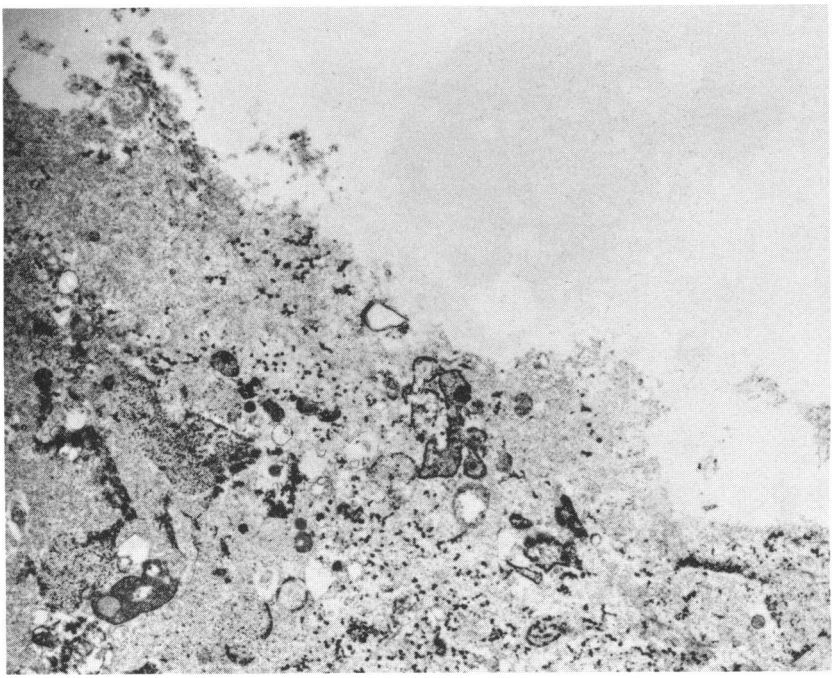

FIGURE 31
Extensive collagen degradation and cellular debris in anterior stroma of central cornea 96 hours after infection (× 12,000).

ness of topical therapy when either fortified concentrations of topical antibiotic solutions or commercially equivalent concentrations of topical antibiotic solution were used. The efficacy of topical fortified gentamicin (20 mg/ml) alone compared with combined topical fortified gentamicin (20 mg/ml) and subconjunctival gentamicin (20 mg) was determined. The topical fortified gentamicin groups and the combined topical-subconjunctival antibiotic groups all showed a good therapeutic effect in three separate six-hour therapy trials using two strains of *Pseudomonas* (Tables VII through IX). Most important, however, gentamicin (20 mg) by the subconjunctival route did not significantly enhance the effectiveness of 20-mg/ml topical gentamicin in any trial with either *Pseudomonas* strain (Tables VII through IX). Also, subconjunctival gentamicin therapy did not improve the effectiveness of topical therapy with gentamicin (20 mg/ml) in a 24-hour therapy trial in rabbits (Table X).

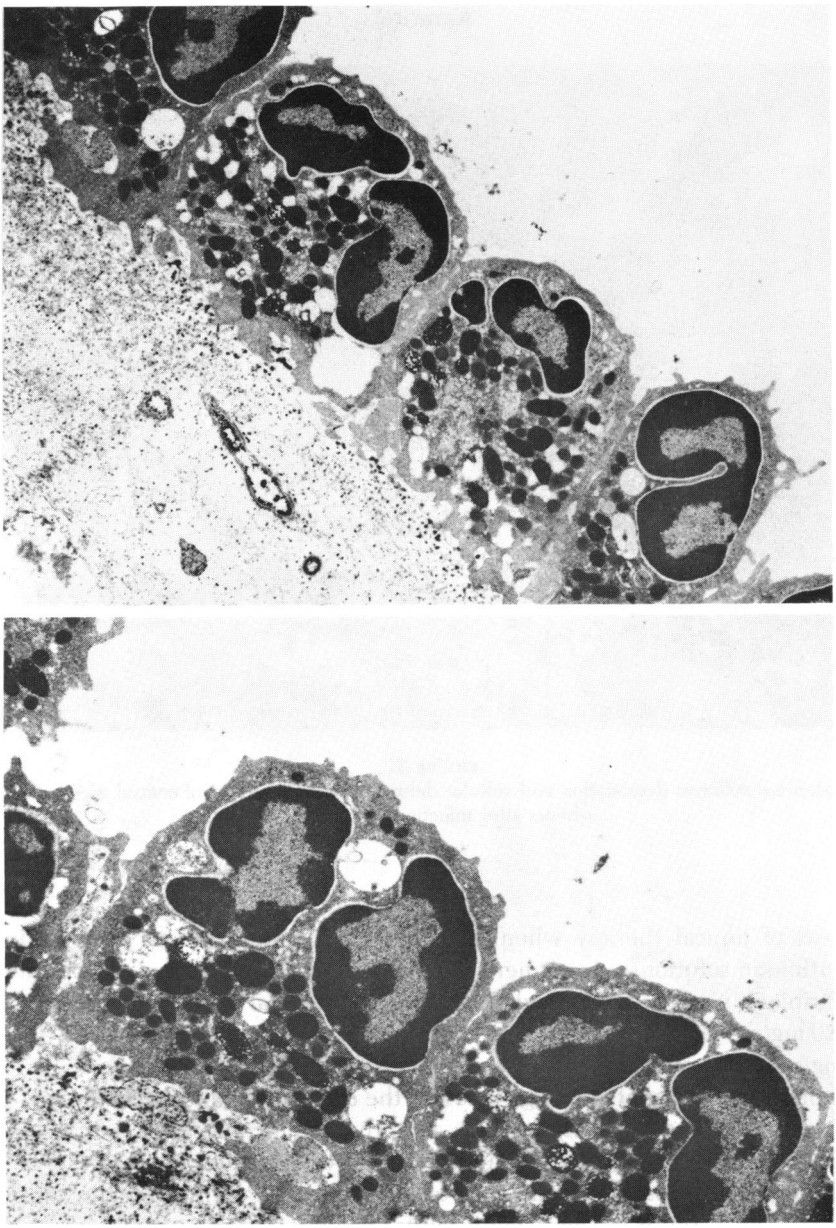

FIGURE 32

Top, PMNs on corneal surface of periphery of ulcer 96 hours after infection (×8,600). Bottom, Micrograph is higher magnification of cell in upper left of micrograph at top (×12,000). Note apparent engulfment of stroma debris by cell process. Collagen degradation is also seen.

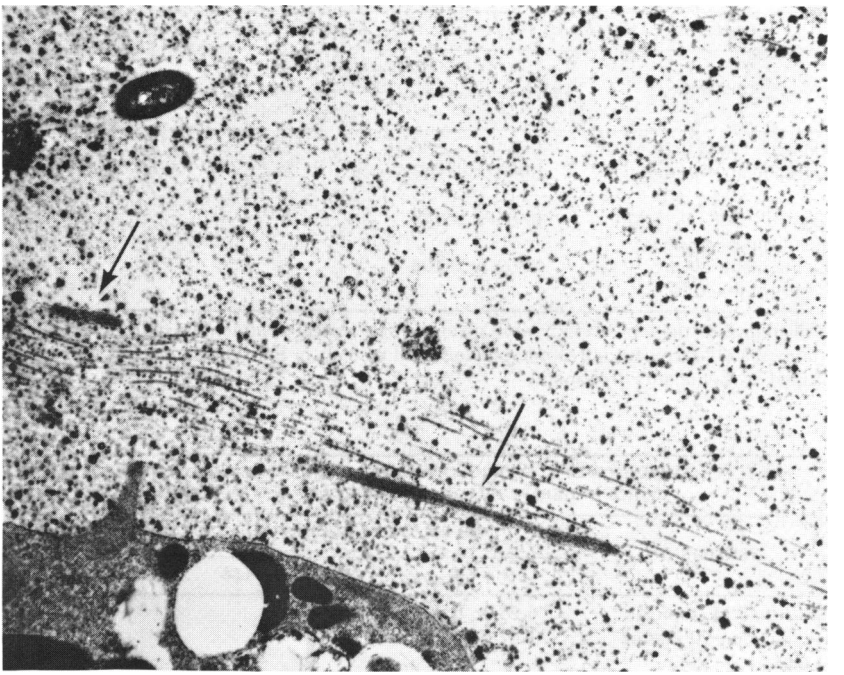

FIGURE 33
Loss of collagen fibers and extensive accumulation of electron-dense particles and electron-dense amorphous material 96 hours after infection deeper in stroma. Tactoid formation (arrows) was observed in many areas. Minor glycocalyx was noted around bacterium (upper left) (×16,600).

TOPICAL VERSUS TOPICAL-SUBCONJUNCTIVAL (COMMERCIAL CONCENTRATION OF GENTAMICIN TOPICALLY) THERAPY TRIAL

Topical gentamicin (3 mg/ml) alone in commercial concentration was compared with combined topical gentamicin (3 mg/ml) and subconjunctival gentamicin (20 mg) therapy in 6-hour and 24-hour therapy trials. The

TABLE II: TOPICAL ANTIBIOTIC CARRYOVER IN RABBITS

TREATMENT*	CORNEAL COLONY COUNTS		P
	$\bar{x}$ ± SD	NO. IN SAMPLE	
Control, NaCl 0.15 M	5.095 ± 0.743	8	.35
Gentamicin, 40 mg/ml	4.549 ± 1.128	8	

*All animals were given a single topical treatment (two drops) and killed immediately for quantitative microbiology.

TABLE III: TOPICAL AND SUBCONJUNCTIVAL ANTIBIOTIC CARRYOVER IN RABBITS

TREATMENT*	CORNEAL COLONY COUNTS $\bar{x} \pm SD$	NO. OF SAMPLES	P
Control, NaCl 0.15 M	4.754 ± 0.086	8	.55
Gentamicin, 40 mg/ml	4.656 ± 0.283	8	
Control, NaCl 0.15 M, subconjunctival	4.865 ± 0.144	8	.59
Gentamicin, 20 mg, subconjunctival	4.832 ± 0.109	8	

*All animals in the topical study were given a single topical treatment (two drops) and killed immediately for quantitative microbiology. All animals in the subconjunctival study were killed six hours after treatment.

TABLE IV: COMPARISON OF SUBCONJUNCTIVAL VS TOPICAL ROUTES USING GENTAMICIN IN SIX-HOUR THERAPY TRIAL WITH *PSEUDOMONAS* 107 IN RABBITS

TREATMENT*	CORNEAL COLONY COUNTS $\bar{x} \pm SD$	NO. OF SAMPLES	P
NaCl 0.15 M, topical	6.28 ± 0.62	6	< .001
Gentamicin, 20 mg/ml, topical	1.47 ± 1.20	6	
Gentamicin, 20 mg, subconjunctival injection at 0 hr OD			.58
OD	1.75 ± 0.75	6	
OS	4.52 ± 1.01	6	

*All treatment was begun 24 hours after infection. All topical therapy consisted of two drops per eye every 30 minutes.

TABLE V: COMPARISON OF SUBCONJUNCTIVAL VS TOPICAL ROUTES USING GENTAMICIN IN SIX-HOUR THERAPY TRIAL WITH *PSEUDOMONAS* 115 IN RABBITS

TREATMENT*	CORNEAL COLONY COUNTS $\bar{x} \pm SD$	NO. OF SAMPLES	P
NaCl 0.15 M, topical	5.71 ± 0.75	6	< .001
Gentamicin, 20 mg/ml, topical	2.08 ± 1.19	6	
Gentamicin, 20-mg, subconjunctival injection at 0 hr OD			.84
OD	2.24 ± 1.46	6	
OS	5.22 ± 1.70	6	

*All treatment was begun 24 hours after infection. All topical therapy consisted of two drops per eye every 30 minutes.

TABLE VI: COMPARISON OF SUBCONJUNCTIVAL VS TOPICAL ROUTES USING GENTAMICIN IN SIX-HOUR THERAPY TRIAL WITH *PSEUDOMONAS* 107 IN RABBITS

TREATMENT*	CORNEAL COLONY COUNTS		P	
	$\bar{x} \pm SD$	NO. OF SAMPLES		
NaCl 0.15 M, topical	4.41 ± 0.72	8		
Gentamicin, 20 mg/ml, topical	0	8	< .001	<.001
Gentamicin, 20-mg, subconjunctival injection at 0 hr OD				
OD	1.40 ± 0.97	8		
OS	4.38 ± 0.74	8		

*All treatment was begun 24 hours after infection. All topical therapy consisted of two drops per eye every 30 minutes.

TABLE VII: COMPARISON OF TOPICAL ROUTE WITH COMBINED TOPICAL/SUBCONJUNCTIVAL ROUTES USING GENTAMICIN IN A SIX-HOUR THERAPY TRIAL WITH *PSEUDOMONAS* 107 IN RABBITS

TREATMENT*	CORNEAL COLONY COUNTS		P	
	$\bar{x} \pm SD$	NO. OF SAMPLES		
NaCl 0.15 M, topical	6.20 ± 0.99	8		
Gentamicin, 20 mg/ml, topical	1.89 ± 1.00	8	< .001	.32
Gentamicin 20-mg subconjunctival injection at 0 hr, OD, and 20 mg/ml topical	1.38 ± 1.03	8		

*All treatment was begun 24 hours after infection. All topical therapy consisted of two drops per eye every 30 minutes.

TABLE VIII: COMPARISON OF TOPICAL ROUTE WITH COMBINED TOPICAL/SUBCONJUNCTIVAL ROUTES USING GENTAMICIN IN A SIX-HOUR THERAPY TRIAL WITH *PSEUDOMONAS* 107 IN RABBITS

TREATMENT*	CORNEAL COLONY COUNTS		P	
	$\bar{x} \pm SD$	NO. OF SAMPLES		
NaCl 0.15 M, topical	5.45 ± 0.71	8		
Gentamicin, 20 mg/ml, topical	2.21 ± 1.16	7	< .001	.39
Gentamicin 20-mg subconjunctival injection at 0 hr, OD, and 20 mg/ml topical	1.83 ± 0.73	8		

*All treatment was begun 24 hours after infection. All topical therapy consisted of two drops per eye every 30 minutes.

TABLE IX: COMPARISON OF TOPICAL ROUTE WITH COMBINED TOPICAL/SUBCONJUNCTIVAL ROUTES USING GENTAMICIN IN A SIX-HOUR THERAPY TRIAL WITH *PSEUDOMONAS* 115 IN RABBITS

TREATMENT*	CORNEAL COLONY COUNTS		P	
	$\bar{x} \pm SD$	NO. OF SAMPLES		
NaCl 0.15 M, topical	5.39 ± 1.04	7		
Gentamicin, 20 mg/ml topical	3.22 ± 0.59	8	< .001	0.26
Gentamicin, 20-mg, subconjunctival injection at 0 hr, OD, and 20 mg/ml topical, OD.				
OD	2.63 ± 1.35	8		
OS (untreated)	6.52 ± 0.90	8		

*All treatment was begun 24 hours after infection. All topical therapy consisted of two drops per eye every 30 minutes.

topical commercial gentamicin groups and the combined topical-subconjunctival antibiotic groups all showed significant and good therapeutic activity compared with saline controls in the two trials. In both trials, the subconjunctival gentamicin (20 mg) significantly enhanced the therapeutic effect of the weaker topical gentamicin (3 mg/ml) commercially equivalent concentration (Table XI and XII). However, the differences were not as significant after the topical antibiotic had been used for 24 hours ($P = .03$).

PERCUTANEOUS-SUBCONJUNCTIVAL THERAPY TRIAL

The next trial was designed to determine whether a percutaneous-subconjunctival (PC-SC) injection (without a conjunctival tract) of gentamicin

TABLE X: COMPARISON OF TOPICAL ROUTE WITH COMBINED TOPICAL/SUBCONJUNCTIVAL ROUTES USING GENTAMICIN IN A 24-HOUR THERAPY TRIAL WITH *PSEUDOMONAS* 107 IN RABBITS

TREATMENT*	CORNEAL COLONY COUNTS		P	
	$\bar{x} \pm SD$	NO. OF SAMPLES		
NaCl 0.15 M, topical	5.57 ± 1.08	8		
Gentamicin, 20 mg/ml, topical	0.33 ± 0.60	8	< .001	.64
Gentamicin 20-mg subconjunctival injection at 0 hr, OD, and 20 mg/ml topical, OD	0.16 ± 0.46	8		

*All treatment was begun 24 hours after infection. All topical therapy consisted of two drops per eye every 30 minutes.

TABLE XI: COMPARISON OF TOPICAL ROUTE WITH COMBINED TOPICAL/SUBCONJUNCTIVAL ROUTES USING COMMERCIAL CONENTRATION OF TOPICAL GENTAMICIN IN A SIX-HOUR THERAPY TRIAL WITH *PSEUDOMONAS* 107 IN RABBITS

TREATMENT*	CORNEAL COLONY COUNTS		P	
	$\bar{x} \pm SD$	NO. OF SAMPLES		
NaCl 0.15 M, topical	6.32 ± 0.86	8	< .001	.003
Gentamicin, 20 mg/ml, topical	3.81 ± 0.93	8		
Gentamicin 20-mg subconjunctival injection at 0 hr, OD, and 20 mg/ml topical, OD	1.89 ± 1.48	8		

*All treatment was begun 24 hours after infection. All topical therapy consisted of two drops per eye every 30 minutes.

(20 mg) would have any therapeutic effect on *Pseudomonas* keratitis in the rabbit. Compared with saline control, the PC-SC gentamicin group demonstrated a significant therapeutic effect when evaluated 24 hours later (Table XIII). A simultaneous routine subconjunctival group, which was not included, would have been valuable for comparison.

PERCUTANEOUS-PERIOCULAR THERAPY TRIAL

Percutaneous-periocular injections of gentamicin (20 mg) through the lower lid were compared with standard subconjunctival injections of

TABLE XII: COMPARISON OF TOPICAL ROUTE WITH COMBINED TOPICAL/SUBCONJUNCTIVAL ROUTES USING COMMERCIAL CONENTRATION OF TOPICAL GENTAMICIN IN A 24-HOUR THERAPY TRIAL WITH *PSEUDOMONAS* 107 IN RABBITS

TREATMENT*	CORNEAL COLONY COUNTS		P	
	$\bar{x} \pm SD$	NO. OF SAMPLES		
NaCl 0.15 M, topical	5.53 ± 1.19	5	< .001	.03
Gentamicin, 20 mg/ml, topical	0.86 ± 0.86	7		
Gentamicin 20-mg subconjunctival injection at 0 hr, OD, and 20 mg/ml topical, OD	0	6		

*All treatment was begun 24 hours after infection. All topical therapy consisted of two drops per eye every 30 minutes.

TABLE XIII: EFFICACY OF PERCUTANEOUS-SUBCONJUNCTIVAL ROUTE (PC-SC) USING GENTAMICIN WITH *PSEUDOMONAS* 107 IN RABBITS

TREATMENT*	CORNEAL COLONY COUNT		P
	$\bar{x} \pm SD$	NO. OF SAMPLES	
NaCl 0.15 M, PC-SC at 0 hr, OU	6.60 ± 0.41	8	.005
Gentamicin, 20-mg PC-SC injection at 0 hr, OD	4.66 ± 1.65	7	

*All treatment was given 24 hours after infection, and the animals were killed for quantitative microbiology six hours after treatment.

gentamicin (20 mg). The subconjunctival injection group showed a good therapeutic effect, whereas the percutaneous-periocular injection group showed a poor therapeutic result even though it was statistically significant (Table XIV). The subconjunctival injection group showed a far greater therapeutic effect than when the injection was given periocularly through the lid into the orbital tissue.

INTRAMUSCULAR THERAPY TRIAL

Intramuscular injection of gentamicin (20 mg) was compred with subconjunctival gentamicin (20 mg). The antibiotic, when given using the intramuscular route, showed no therapeutic effect, whereas the subconjunctival antibiotic had a good therapeutic effect (Table XV).

TABLE XIV: COMPARISON OF SUBCONJUNCTIVAL AND PERIOCULAR INJECTION ROUTES USING GENTAMICIN WITH *PSEUDOMONAS* 107 IN RABBITS

TREATMENT*	CORNEAL COLONY COUNTS			P
	$\bar{x} \pm SD$	NO. OF SAMPLES		
NaCl 0.15 M, subconjunctival at 0 hr, OU	5.85 ± 0.83	8	.02	< .001
Gentamicin, 20-mg periocular injection at 0 hr, OD	4.50 ± 1.02	6	< .001	
Gentamicin, 20-mg subconjunctival injection, OD	1.26 ± 0.70	6		

*All treatment was given 24 hours after infection. The animals were killed for quantitative microbiology 24 hours after treatment.

TABLE XV: COMPARISON OF SUBCONJUNCTIVAL WITH INTRAMUSCULAR INJECTION ROUTES USING GENTAMICIN IN A SIX-HOUR THERAPY TRIAL WITH *PSEUDOMONAS* 107 IN RABBITS

TREATMENT*	CORNEAL COLONY COUNT		P
	$\bar{x} \pm SD$	NO. OF SAMPLES	
NaCl 0.15 M subconjunctival, 0 hr, OU	6.66 ± 0.49	6	.82
Gentamicin, 20-mg, intramuscular injection at 0 hr	6.59 ± 0.44	8	
Gentamicin, 20-mg subconjunctival injection at 0 hr, OD			< .001
OD	2.15 ± 0.80	8	< .001
OS (untreated)	6.19 ± 0.55	8	

*All treatment was given 24 hours after infection. Animals were killed for quantitative microbiology six hours after treatment.

DISCUSSION

SEQUENTIAL ELECTRON MICROSCOPY STUDIES

The results of this sequential ultramicroscopic study added some new concepts, clarified certain aspects of the pathogenesis of *Pseudomonas* keratitis that were discussed previously, and indicated areas for further investigation.

CHARACTERISTICS OF SUPERFICIAL INJURY KERATITIS MODEL

The experimental keratitis-superficial injury model used in this study was different from previous intrastromal injection models studied ultramicroscopically, in which organisms were injected intrastromally. The present model is more similar to human keratitis in the mode of onset after superficial injury and contamination and in the sequence of events following the induction with analogous early superficial infiltration, ulceration, and anatomic spread of corneal disease.

The experimental keratitis model was uniform and highly reproducible, with an infection rate of 97% to 100%. The high infection rate was dependent on combined injury to both epithelium and anterior stroma, and on inoculation of undiluted growths of the strains of *Pseudomonas* used. If only epithelium was injured or diluted suspensions of *Pseudomonas* were utilized, or both, the infection rate dropped (Table I). It has been shown

that adhesion to cells and tissue is important in *Pseudomonas* infections.[12] An explanation may be that there was probably more adhesion or more bacteria adherent to injured corneal stroma than to injured epithelial cells (Fig 8), and stromal penetration may be facilitated without basement membrane or Bowman's layer (Fig 11).

INITIAL ADHESION AND INVASION OF ORGANISM

Pseudomonas infections in the rabbit cornea probably begin with the specific adhesions of certain strains to injured cells or tissues as seen in the early hours of infection (Fig 8, 10, and 11). Adhesion to intact epithelial cells was poor since only an occasional organism was seen on intact, uninjured epithelial cells. An intact, healthy epithelium probably functions not only as a barrier to organisms like *Pseudomonas*, but also as a vertically placed, "nonadherent" surface or "slippery walkway" for most bacteria. Movement of the upper lid across a healthy nonadherent surface may contribute to the kinetics. *Corynebacterium diphtheriae*, *N gonorrhoeae*, and possibly *Hemophilus* may penetrate (and would first have to adhere to?) intact epithelium and cause corneal infection, but corneal infection caused by most other bacteria (including *Pseudomonas*) has been uncommon in a normal uninjured cornea. Once the corneal epithelium or stroma or both have been compromised, bacteria may adhere and infect. In some instances, the nonadherent function may be even more important than that barrier function. Infection may occur in corneas that have abnormal superficial epithelium and only superficial cell erosions, without a full-thickness loss of the epithelial barrier. In these corneas, the abnormal superficial cells probably provide lack of a nonadherent surface. Once the bacterium is able to adhere, the environment may be provided within which bacterial toxins and enzymes diffuse to attack cells and tissues and permit penetration. After superficial injury to epithelium and stroma, penetration and invasion of normal stroma by *P aeruginosa* was seen as early as one hour after infection (Fig 11 and 12).

SLIME ENVELOPE (GLYCOCALYX)

In the early stages of infection, a prominent electron-lucent envelope was seen around the bacteria. This may represent the slime envelope or glycocalyx, which has been reported to provide adhesion properties and inhibit phagocytosis by PMNs.[12] The envelope was present early when it apparently was needed most for its biologic activity. Twenty-four hours later, most of the envelope had disappeared, although a minor glycocalyx was still seen about some organisms at 96 hours (Fig 33). Dogget[43] has recently pointed out, in writing about the adaptability of *P aeruginosa*,

that "the ability of these microorganisms to mutate or change their metabolic behavior to adapt to the environment for survival purposes no doubt reflects one of the more efficient gene pools awaiting human inquiry." Further study of this envelope both in vitro and in vivo will be necessary to clarify some of our initial concepts.

EVENTS IN PATHOGENESIS OF BACTERIAL INFECTION

Jones[40] has summarized the sequence of events in a bacterial infection of a target organ such as the cornea, and included the following: (1) entry of organism, (2) multiplication, (3) spread of organism, (4) host inflammatory response, (5) encounter of the organism with the phagocyte cell, (6) host immune response, (7) tissue damage, and (8) tissue repair and recovery. Perhaps adhesion should be added before the initial entry of the organism. Besides the experimental observations in the cornea in this study and in other tissue systems,[11,12] the mechanism also makes sense conceptually. As for the pathogenesis of a corneal infection, it is reasonable to assume that many bacteria would have to adhere to a cornea to permit the subsequent penetration, entry, and invasion. If this assumption is correct for many bacteria, it may have prophylactic therapeutic implications if adherence can be altered chemically. Further study in this area will be interesting.

PMN RESPONSE IN KERATITIS

Source of PMN.—PMN infiltration was seen as a result of the superficial injury, but greatly enhanced by bacterial infection. Robb and Kuwabara[128] have emphasized the importance of the tear film as an important carrier for inflammatory cells after superficial corneal wounding. Probably the tear film in the present model also played an important carrier role. PMNs were not seen anywhere on the epithelial surface with SEM or TEM, but the infected wound was heavily infiltrated with PMNs at four hours, and the infected anterior stroma was infiltrated with PMNs at eight hours. PMN infiltration also started at four to six hours in published studies of wounded, uninfected rat and rabbit corneas.[128,129] Early in the infection in this model, PMNs moved into the infected stroma almost entirely from the infected ulcerated defect. Movement of the PMNs was from the central ulcer toward the limbus. The PMN contribution to stromal inflammation from the limbus became prominent at 48 hours. By 72 hours there was a continuous zone of stromal PMNs, with likely PMN participation by then from both the limbus and the ulcerated corneal

surface. Evidently the contribution of PMNs to the latter came mainly from the tear film[128] (Fig 30 and 32).

PMN-phagocytosis and digestion.—Phagocytosis was noted as an important defense mechanism, and bacteria were probably digested by lysosomal enzymes. PMN phagocytosis has been emphasized as perhaps the most important defense mechanism in *Pseudomonas* infections,[31] and seemed true in *Pseudomonas* keratitis. It has been recently reported, in regard to *Pseudomonas* keratitis in animals that were made PMN-deficient, that the corneal host defenses were significantly impaired (when microbial killing was compared to controls).[35]

Many bacteria were phagocytized by PMNs, and undergoing digestion within them from four hours on after initiation of keratitis (Fig 21, 22, and 27). A review of this process indicated that rapid degranulation occurs after phagocytosis of organisms, as cytoplasmic granules fuse with the phagocytic vacuole and discharge their bactericidal proteins and hydrolases.[130] Myeloperoxidase (MPO) and lysozyme (LZM) are PMN granule enzymes reported to have specific bactericidal activity.[131] A recent study has demonstrated that significant MPO and LZM enzyme levels were found in both human and rabbit PMNs,[42] and were probably responsible for destruction of the phagocytized bacteria noted in this study. Although rabbit and human PMNs differ morphologically and possibly functionally, the mechanism of bactericidal effect after phagocytosis may be similar since the same bactericidal enzymes are present in both rabbit and human PMN granules. MPO, in association with H_2O_2 and oxidizable cofactors, probably constitutes a predominant mechanism of microbial killing in other infections,[132] and may play an extremely important role in host defense in bacterial keratitis.[40]

PMN role in corneal damage.—As seen in *Pseudomonas* keratitis, PMNs are protective and an important defense mechanism, but they are a double-edged sword: they also damage host cell tissue. Previous electron microscopic studies of *Pseudomonas* keratitis were reviewed in the background material.[4,5,33,37,76] In experimental keratitis using live *P aeruginosa* organisms and intrastromal inoculation, extensive collagen damage was noted and the collagen breakdown was thought to be due primarily to host-cell PMN.[5,37] The present study, using a superficial injury model, demonstrated probable early localized collagen degradation at 8 and 16 hours after infection. Extensive loss of collagen fibrils was observed 24 hours after infection, with viable *P aeruginosa*, and progressed in the later hours of infection. The ultrastructural changes seen in these rabbit corneas were similar to changes described by Van Horn et al in guinea pig cornea[5] and by Rowsey and Nisbet[36] in rabbit cornea after inoculation of

concentrated PMN lysosomal preparations. Collagen fibrils were replaced with amorphous desposits of electron-dense material, and tactoidal formation occurred. As previously mentioned, tactoidal formation has been reported to be associated with in vitro studies of collagen degradation.[77] Extensive collagen degradation has also been reported in a study of human *Pseudomonas* keratitis.[76]

Collagen breakdown in the current study was always associated with the presence of PMNs in the first 48 hours of infection. However, at 96 hours there was widespread collagen breakdown which, in many areas, was not associated with either PMNs or bacteria. It was assumed that PMNs had already been there and had also broken down.

Gray and Kreger[4] did not observe collagen breakdown in their observations at only 24 hours after infection. They described a loss of the web-like pattern of proteoglycan ground substance and the dispersal of ultrastructurally normal collagen, using a different strain of *P aeruginosa*. Brown et al[23] have demonstrated that some strains of *Pseudomonas* produced an enzyme that, when isolated and injected into corneal stroma, may liquefy cornea. Kessler et al[24] emphasized that in *Pseudomonas* keratitis, corneal destruction depends not only on proteases of *Pseudomonas* but on host-derived (PMN) enzymes. Enzymes from both organisms and PMN can degrade both corneal collagen and corneal proteoglycan. Also, different strains of *P aeruginosa* are highly adaptive and display different invasive properties and enzyme production.[43] Some proteases or nonspecific collagenases of *Pseudomonas* organisms may require the loss of the protective corneal proteoglycan, also induced by either an organism or host cell, before corneal collagen can be broken down.[41]

The gradual and progressive loss of epithelium associated with a spreading ulcer may be explained by the loss of epithelial basement membrane in the inflamed area of an infected corneal ulcer. Most likely, PMN enzymes (Fig 23), or possibly bacterial enzymes, may be responsible for loss of epithelial basement membrane and subsequent peel or lysis of epithelium. Sliding of the epithelium was not thought to be a mechanism in the areas observed with multilayered epithelium with microvilli.

The role of host-cell PMN in corneal damage was confirmed in this study. The role of *Pseudomonas* enzymes and toxins must also be important with certain strains, but this has been difficult to study because of host-cell response and quantitative and qualitative variables in the bacterial products evaluated. Ohman et al[28] have recently employed an ingenious approach by preparing and comparing parental strains with genetic mutants deficient in exotoxin A and elastase. They evaluated their effect on the cornea clinically. Mutants lacking many toxins and enzymes can be

prepared. The technique is probably the best currently available approach for determining the relative importance of the role of a given toxin or enzyme in the corneal damage of a corneal infection. Further research should be carried out, comparing parental strains and deficient mutants, using both clinical and ultramicroscopic techniques.

EXPERIMENTAL KERATITIS-THERAPY STUDIES

AMINOGLYCOSIDES

The aminoglycosides, gentamicin and tobramycin and possibly amikacin, have similar properties in vitro and in vivo[46] and probably would act similarly when using topical, subconjunctival, periocular, or intramuscular routes in this experimental model. Mechanisms of action in their antimicrobial activity and their similarities and principles regarding antibiotic resistance were reviewed in the introduction.

TOPICAL THERAPY

Topical antibiotic alone or subconjunctival antibiotic alone were found to be superior in the treatment of experimental *Pseudomonas* keratitis in rabbits compared with percutaneous-periocular antibiotic alone or systemic antibiotic alone, which had little or no significant therapeutic antimicrobial effect. The efficacy of topical fortified gentamicin (20 mg/ml) alone was equal to subconjunctival gentamicin (20 mg) in six-hour therapy trials, but topical antibiotic was therapeutically superior in a 24-hour trial. There has been general agreement that certain topical antibiotics, especially topical fortified aminoglycoside solutions, have been extremely effective in treating *Pseudomonas*[78,101,104,105,108-110] and other forms of bacterial keratitis.[133] The efficacy of fortified topical gentamicin has been confirmed in the current studies.

SUBCONJUNCTIVAL THERAPY

Subconjunctival aminoglycosides and other antibiotics have been known to penetrate into aqueous or corneal tissue[115,119,120,134-136] and reported to have a clinically therapeutic effect in experimental bacterial keratitis.[101,103,106,107,112] However, the quantitative microbiologic effect of subconjunctival aminoglycosides has been unclear, and their relative efficacy and value have been questioned, compared with fortified topical regimens.[110,119,122,123] Also, their role as therapy adjunctives to an intensive topical regimen has been unclear. The data in the present multiple therapy trials show that aminoglycoside antibiotic, when given subconjuncti-

vally, had a definitively good therapeutic effect in this superficial injury model in repeated trials (Tables IV through VI, XIV, and XV); this is opposed to previous reports in which subconjunctival aminoglycosides showed little or no therapeutic result against the microbial population.[110,123] Both groups of investigators used the intrastromal injection model in which the pharmacokinetics were probably different, especially because of initial ulceration in the current model. The early surface defect, as in human keratitis, may be important pharmacokinetically. Probable mechanisms will be reviewed.

MECHANISMS OF ACTION IN SUBCONJUNCTIVAL THERAPY

Mechanisms of corneal penetration of antibiotics after a subconjunctival injection probably include both direct diffusion and leakage from the site acting as a depot for topical corneal penetration, according to these studies. Subconjunctival antibiotic presumably arrives at the cornea from the leak through the conjunctival tract and possibly also through diffusion. Greater early access of leaking antibiotic through the initial ulcer to the cornea in this superficial trauma keratitis model may be one reason for the good therapeutic effect obtained with subconjunctival antibiotics and may be more analogous to the pharmacokinetics in human beings.

The study of the PC-SC antibiotic route gives some insight into the above mechanisms. The PC-SC route showed a therapeutic result without the benefit of an anatomic conjunctival leakage tract (Table XIII), and penetration to the cornea was probably a combination of direct diffusion and ooze through even intact conjunctiva, which will be discussed. A simultaneous comparison with routine subconjunctival antibiotic injection was not done, and it would have been informative. However, nonsimultaneous comparisons with the other subconjunctival trials showed a much greater therapeutic benefit with a standard subconjunctival injection.

The only perceivable difference between the PC-SC injection and the SC injection was an absence of a conjunctival tract to permit more leakage in the former. Even though no visible leakage was seen during routine SC injections around the needle tract, experiments with similar injections of fluorescein (10%) to evaluate conjunctival leak demonstrated a minute leakage around the tract, and fluorescein was seen in the tear film 30 minutes later. Also, when fluorescein (10%) was injected using uneventful PC-SC injections (without conjunctival tracts), fluorescein was noted in the tear film a few minutes after successful PC-SC injections and 30 minutes later in the tear film, indicating diffusion of fluorescein, most likely through anatomically intact conjunctiva.

If the assumptions are correct that mechanisms for the therapeutic effect in subconjunctival therapy include diffusion and drug provided from the "antibiotic leak" from the subconjunctival depot, the difference in therapeutic effect between previously reported intrastromal injection models in which the therapeutic effect was poor, and the present model in which it was good, may be partially explained. Perhaps there was a greater access of leaking antibiotic to the cornea through the superficial wound in the present model than there was in the intrastromal injection model. In that case, the data in the present studies would have more although indirect, application to the human model since *Pseudomonas* keratitis induced in the present studies is probably more analogous to most cases of human *Pseudomonas* keratitis than the intrastromal injection model.

Another explanation for the relative lack of a good therapeutic result in studies using the intrastromal model could be the fact that this model is a more severe test of therapeutic efficacy, and therapeutic effect may have been more difficult to detect. This may be the case in one of the published reports,[110] but conclusions cannot be drawn from only an abstract in the other report.[123] Ultimately, however, the mechanical or physiologic mechanisms that account for the differences in response to therapy between investigators and studies in different laboratories are not always known.

VALUE OF SUBCONJUNCTIVAL THERAPY AS ADJUNCT

An important question with clinical ramifications is this: Does subconjunctival antibiotic usually enhance the therapeutic effect of a frequent, topical, fortified antibiotic regimen enough to warrant the side effects of subconjunctival antibiotics? Animal studies sometimes modify clinical views, and they have changed my views on the relative merit of using certain subconjunctival antibiotics in certain corneal ulcers.

In the current studies, and in multiple trials, subconjunctival antibiotic did not enhance the therapeutic effect of topical antibiotic regimens when fortified antibiotic (gentamicin, 20 mg/ml) was used topically (Tables VII through X). However, when only lower concentrations of topical antibiotic, ie, gentamicin (3 mg/ml) were used, which is the equivalent of the commercial concentration available, SC antibiotic enhanced the therapeutic effect (Table XI and XII).

Sloan et al[119] demonstrated that extremely high concentrations of gentamicin are achieved in the aqueous of rabbits with *Pseudomonas* keratitis after either frequent topical therapy with fortified gentamicin (20 mg/ml), subconjunctival gentamicin (20 mg), or continuous-lavage gentamicin.

They suggested that frequent topical medication be used as a more logical and less traumatic route than subconjunctival administration. The therapeutic studies herein reinforce their recommendation. Baum et al[120] reported higher gentamicin concentrations after subconjunctival injection as compared with topical gentamicin, but they used topical gentamicin (3 mg/ml).

Subconjunctival antibiotic may be worthwhile as an adjunct to topical therapy, if the topical regimen is not ideal, that is, if frequent topical application is not possible logistically or fortified topical antibiotic solutions are not available. Topical fortified gentamicin or tobramycin should probably be considered the mainstay of therapy in the treatment of *Pseudomonas* keratitis when it can be administered initially at least every 30 minutes.

Keratitis present at 24 hours after infection was the standard keratitis treated in these therapy trials. The infection at 24 hours was moderately severe, but therapeutic trials should also be performed at later states, ie, at 48 hours when the keratitis is exceedingly severe. Would subconjunctival antibiotic enhance ideal topical antibiotic therapy in severe keratitis in rabbits (Fig 4C and 4D)? We do not know the answer, but further studies may be helpful. Many clinicians have recommended, and may continue to recommend, subconjunctival antibiotics at the first treatment, before the cause is known, as well as in severe keratitis with extensive deep infiltration, on the basis of insight from penetration data. This makes sense to me until we have more reliable information from further experimental and clinical observations in the extremely severe cases of keratitis.

The role of subconjunctival antibiotics in the initial treatment of *Pseudomonas* keratitis, and the past traditional emphasis on repeated daily or twice-daily subconjunctival injections as adjunctive therapy, need further evaluation clinically, considering the data in the present studies, the clinical side effects, and the risks of subconjunctival antibiotics. I have modified my views and now only infrequently recommend subconjunctival antibiotic injections in the treatment of most cases of bacterial keratitis. Also, Wilson[2] had previously recommended SC antibiotic therapy as adjunctive treatment for all types of bacterial keratitis. However, he has abandoned the use of SC injections, has had excellent results in his treatment of bacterial keratitis with fortified topical antibiotic solution given every 30 minutes, and now does not recommend SC antibiotic therapy in most forms of bacterial keratitis (personal communication, January 7, 1981). I believe the relative value of routine adjunctive SC antibiotics in *Pseudomonas* keratitis, and probably in other causes of

bacterial keratitis, is even more questionable in the light of the above information.

CLINICAL CORRELATION

A retrospective clinical study of 65 cases of suspected bacterial keratitis from January 1978 to November 1980 was recently carried out by reviewing records from our Ocular Microbiology Laboratory, at the Medical College of Wisconsin. There were 45 cases of bacterial keratitis with positive corneal cultures. Cases in which bacteria were seen on Gram-

TABLE XVI: BACTERIAL KERATITIS 1978-1980 (N = 45)

ORGANISM	NO. OF ISOLATES		
	SINGLE INFECTION (N = 40)	MIXED INFECTION (N = 5)	TOTAL
Gram-positive Cocci			
Staphylococcus aureus	14*	2	16
Staphylococcus epidermidis	5	2	7
Streptococcus pneumoniae	4	1	5
α-Streptococcus-viridans group	2	1	3
β-Streptococcus	0	2	2
			33
Gram-positive Rods			
Bacillus sp	1	0	1
Propionibacterium sp	0	1	1
			2
Gram-negative Cocci			
Neisseria gonorrhoea	1	0	1
			1
Gram-negative Rods			
Pseudomonas aeruginosa	8	0	8
Klebsiella pneumoniae	2	0	2
Proteus sp	0	1	1
Moraxella sp	1	0	1
Haemophilus sp	1	0	1
Eikenella corrodens	1	0	1
			14
Total	40	10	50

*Includes one isolate of *S aureus* mixed with *Candida albicans*.

stained material from corneal scrapings but which had negative culture results, were not included. Bacterial cultures were taken by the same techniques as those also used at the Francis I. Proctor Laboratory, San Francisco, and recently described.[72] Of the 45 cases of bacterial keratitis seen and supervised by the author from 1978 to 1980, eight cases (18%) were caused by *P aeruginosa* (Table XVI). A review of the *Pseudomonas* cases showed that seven of eight cases received an initial regimen of fortified gentamicin (20 mg/ml) topically every 30 minutes, with or without adjunctive subconjunctival antibiotics. The two earliest cases received the topical fortified gentamicin regimen plus multiple SC injections of gentamicin (20 mg); the three following cases received the topical fortified gentamicin regimen plus one initial SC gentamicin (20 mg); and the last two, most recent, cases have received only frequent topical fortified gentamicin therapy. All cases responded rapidly to treatment. The two most recent patients, who received topical fortified gentamicin alone, are seen in Figures 34 and 35 before and after therapy. The results of using the topical therapy alone were impressive, but further experience is necessary before stronger recommendations can be made.

PERCUTANEOUS-PERIOCULAR AND SYSTEMIC THERAPY

Percutaneous-periocular antibiotic therapy probably has no place in the treatment of *Pseudomonas* keratitis. The antibiotic injection site may locate in various orbital tissues, including orbital fat, and may have little or no more preferential ocular penetration potential than when the injection is given in a site much more distant from the eye, ie, the gluteal muscle.

Intramuscular antibiotic therapy had no effect even though an extremely high dose of gentamicin was used in the rabbit compared with the intramuscular dose that could be used in a human being. Similar results have been reported with intramuscular tobramycin in rabbits.[110] However, intravenous antibiotic, the form of systemic antibiotic most commonly used as adjunctive therapy by some, was not evaluated, so more relevant conclusions cannot be made with regard to systemic antibiotic therapy. My views were previously reviewed in the introductory remarks regarding systemic antibiotic therapy, and the current systemic study did not modify my views.

CRYOTHERAPY-ANTIMICROBIAL ACTIVITY

An interesting approach to the antimicrobial treatment of experimental *Pseudomonas* keratitis has recently been reported. Cryotherapy was used

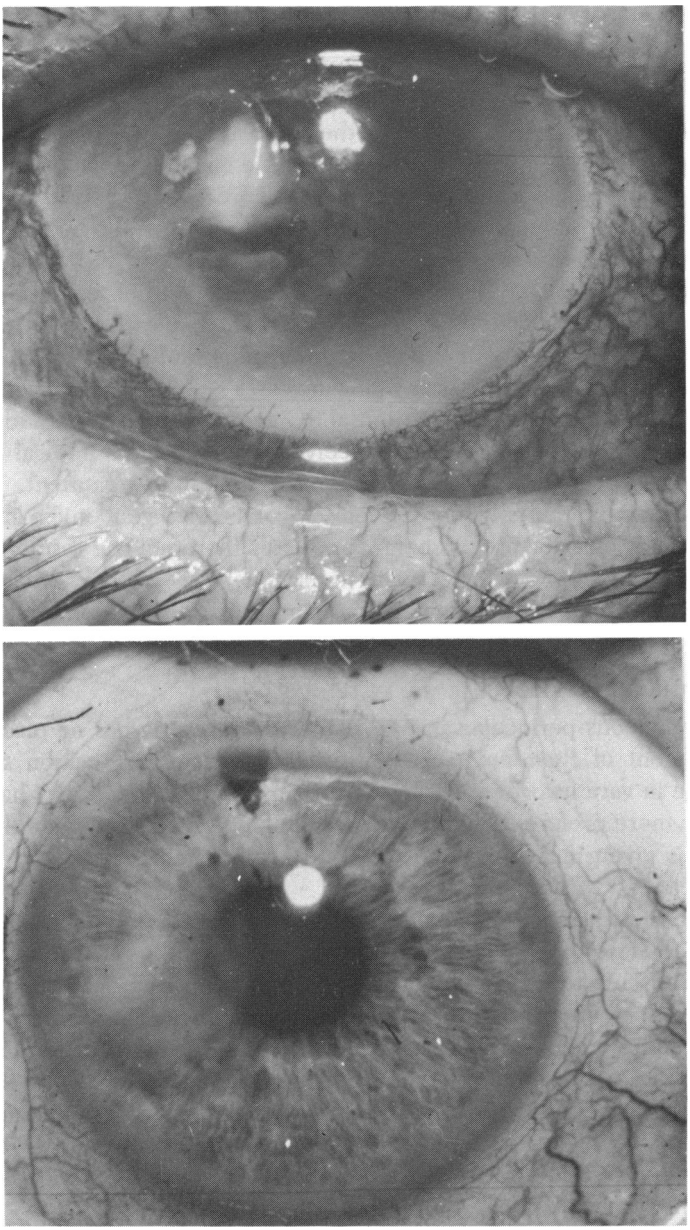

FIGURE 34

Moderately severe *Pseudomonas* keratitis with rapid onset in 73-year-old man, induced while wearing aphakic soft lens. Top, Pretreatment visual acuity was HM at six inches. Bottom, Posttreatment visual acuity after administration of frequent topical fortified gentamicin solution, was 20/30+.

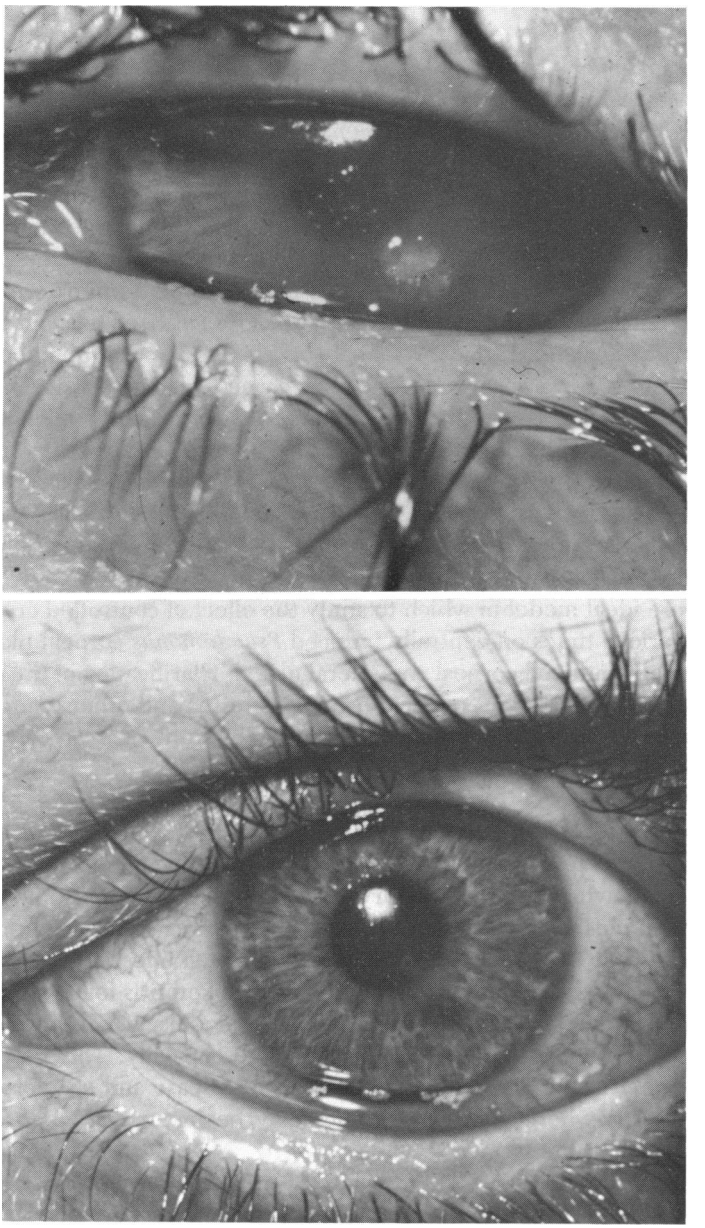

FIGURE 35

Moderately severe *Pseudomonas* keratitis with rapid onset in 19-year-old woman while wearing cosmetic soft contact lens. Top, Pretreatment visual acuity was HM at 1 ft. Bottom, Posttreatment visual acuity, after administration of frequent topical fortified gentamicin solution, was 20/25.

in *Pseudomonas* keratitis and demonstrated a marked bactericidal effect in vivo by killing viable corneal bacteria.[137] It has a rapid bactericidal effect when used alone in *Pseudomonas* keratitis, and it also significantly potentiated topical antibiotic therapy for most *Pseudomonas* strains tested. However, the whole cornea was frozen, which was necessary for therapeutic effect in their studies, since the intrastromal injection model was used; in this model, organisms diffuse immediately to the limbus even though a localized central corneal injection was administered. The technique of cryotherapy would have to be more localized to avoid significant corneal toxicity. It may eventually have special application in certain specific cases, ie, in localized peripheral corneal ulcers and also in *Pseudomonas* keratitis where there is secondary scleral involvement that is notoriously difficult to treat clinically. There has been a recent report of the use of cryotherapy clinically in a few patients with severe uncontrolled *Pseudomonas* keratitis.[138] However, the medical and surgical treatments used clouded whether cryotherapy of itself was really beneficial. The present model of *Pseudomonas* keratitis used in this study would be a more ideal model in which to study the effect of controlled cryotherapy at various times on centrally induced *Pseudomonas* corneal ulcers or peripherally induced corneal and scleral ulcers. Clarification of the role of cryotherapy in treatment of *Pseudomonas* keratitis and other gram-negative bacterial corneal infection requires further study.

SUMMARY

This thesis presents a simple rabbit model of *Pseudomonas aeruginosa* keratitis following superficial injury arrived at after using multiple induction techniques. The model was highly reproducible in terms of uniform corneal disease that was progressive and had a high infection rate (97% to 100%). The model mimics human *Pseudomonas* keratitis following superficial injury more than intrastromal injection models, which have previously been evaluated clinically and ultramicroscopically. This superficial model is better for evaluating mechanisms of disease and also for use in therapy trials where pharmacokinetics may be important because it is more similar to the human disease.

The superficial injury-*Pseudomonas* keratitis model was evaluated in this thesis sequentially with scanning electron microscopy (SEM), transmission electron microscopy (TEM), and light microscopy and correlated with clinical biomicroscopic observations. Minutes after infection, SEM and TEM demonstrated large numbers of organisms selectively adhering to the injured corneal epithelium and stroma, as opposed to a relative

nonadhesion of *Pseudomonas* organisms to the intact uninjured corneal epithelium—the "slippery walkway." The injured tissue or cell may also play a role in biologic adhesion. The electron-lucent envelope around the pseudomonads probably represented a slime envelope or glycocalyx, which has recently been reported to provide adhesion to tissue surfaces. The biologic adhesive property of the envelope of certain bacteria has just recently been emphasized as important in certain bacterial infections, ie, *N gonorrhoeae* in urethritis, *P aeruginosa* in cystitis. The adhesive phenomenon is probably the initial important mechanism in *Pseudomonas* keratitis and necessary before penetration and invasion of the organism can occur. The results of this study open a new area for research, not only in *Pseudomonas* keratitis, but in other forms of bacterial keratitis. Certain bacteria may require a "biologic adherence" to exert their biologic activity in vivo and produce disease. Either the bacterium may provide the adherence, certain injured tissues may contribute, or most likely in certain diseases, both organisms and host tissues—injured or uninjured—may play a role.

The organisms after adherence penetrated corneal stroma within one hour after infection, and spread radially through corneal stroma peripherally and deep to the entry site. The organisms were followed by many PMNs within four hours after infection. In the first 24 hours the PMN contribution to the corneal disease was primarily from the tear film as opposed to a limbal-stromal-central ulcer PMN movement. In the early hours of infection, the PMNs selectively adhered to the superficial injury site and not to intact corneal epithelium. The limbal-stromal contribution of PMNs to the corneal disease was not prominent until 48 hours after infection. This model may be more susceptible to topical therapeutic PMN inhibitors, and a better model with which to evaluate them than the intrastromal model, since PMNs arrive mainly through the tear film early in the infection.

The role of host-cell PMN was most important in corneal destruction with the strain used in this study. However, we know from other studies that the role of products of the organism *P aeruginosa* was important in producing corneal disease. The role of bacterial toxins and enzymes, however, is probably best evaluated using the ingenious technique of comparing parent *Pseudomonas* strains with genetically deficient, mutant daughter strains lacking specific known toxin or enzyme activities. The approach may provide a more effective means of evaluating comparatively the relative important of a given toxin or enzyme in corneal damage. However, besides the excellent clinical observations made by those investigators, comparative ultramicroscopy would be valuable in further

evaluating various parental and mutant genetically deficient strains.

The phagocytic cell, primarily the host PMN, is probably the main host defense against nontreated *Pseudomonas* organisms in the cornea, as seen in this study, and is also reported in systemic infections.

It was clear in the multiple controlled studies presented in this thesis that subconjunctival (SC) gentamicin had a definite therapeutic effect in multiple trials with the *Pseudomonas* strains used. However, SC antibiotic as adjunctive therapy did not enhance the therapeutic effect of a fortified topical antibiotic regimen when higher concentrations of gentamicin sulfate solution (20 mg/ml) were used for frequent topical treatment. However, when a topical regimen of the weaker commercially equivalent concentration (3 mg/ml) was used, SC gentamicin was useful as adjunctive therapy. This study indicated that the main pharmacokinetic mechanism for the therapeutic effect of a paralimbal SC antibiotic injection is probably the conjunctival "leak" from the injection site acting as a depot, although direct diffusion may play a role. Further investigation in this area is needed.

Percutaneous-periocular antibiotic treatment showed a poor therapeutic result compared with standard paralimbal SC treatment of the keratitis. Intramuscular antibiotic treatment showed no therapeutic effect.

Preliminary data in the treatment of cases of human *Pseudomonas* keratitis with frequent, topical, fortified gentamicin solution alone—without adjunctive SC or systemic therapy—were presented, and demonstrated a rapid therapeutic effect in each patient. Further experience is necessary before strong recommendations can be made.

Research should answer questions and, in some cases, solidify or modify the views of the investigator and colleagues. Research may also raise questions for further investigation. I hope these studies have accomplished both.

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