

The pathogenesis of bacterial keratitis: studies with *Pseudomonas aeruginosa*

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Suzanne MJ Fleiszig* MScOptom PhD
FAAO

David J Evans*† PhD

* School of Optometry, University of
California, Berkeley, CA, USA

† Touro University College of
Osteopathic Medicine, Vallejo, CA, USA

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Bacterial keratitis is a sight-threatening corneal disease that is most commonly associated with the extended wear of soft contact lenses. Over the past decade, we have investigated the pathogenesis of infectious keratitis involving the opportunistic pathogen *Pseudomonas aeruginosa*. Our research has focused on understanding the respective roles of bacteria and host in the establishment of this infection. Here, we provide a current perspective on *P. aeruginosa* keratitis, reviewing some of the research developments that have helped shape our views on the mechanisms by which pathogen and host response cause corneal disease. *P. aeruginosa* may provide a model for the pathogenesis of bacterial keratitis and help further elucidate the complex array of host factors that normally protect the cornea from infectious agents.

Key words: cornea, epithelium, keratitis, pathogenesis, *Pseudomonas aeruginosa*, tears

Bacterial infection of the cornea (Figure 1) is considered a relatively rare but serious medical condition requiring urgent medical attention because of the potential for reduced vision or even vision loss in the affected eye(s). Predisposing factors for infectious keratitis include the extended or overnight wear of soft contact lenses;^{1,2} ocular surgical procedures, for example, LASIK;³ ocular disease and ocular injury.⁴ Bacteria isolated from patients with keratitis include a variety of gram-positive and gram-negative organisms such as *Staphylococcus* spp., *Streptococcus* spp., *Pseudomonas* spp. and *Serratia* spp. Infecting bacteria are likely to be derived from environmental sources, patients' normal skin and nasopharyngeal flora, contact lens care solutions or lens cases, topical drug or irrigation solutions or ocular instruments. Continued incidence of infectious keratitis,^{1,2,4} increasing antibiotic

resistance among clinical isolates⁴ and the threat of reduced or loss of vision necessitate continued research investigation into the pathogenesis and treatment of this disease. Given that the eye is regularly exposed to the infecting bacteria and many other microbes on a daily basis and yet does not develop corneal infection, the major questions that need to be answered to develop effective strategies for prevention and treatment of infectious keratitis include:

1. what virulence factors do bacteria use to establish and maintain corneal infection?
2. what are the defences of the healthy cornea that allow resistance to infection by microbial pathogens?
3. how do predisposing factors such as contact lens wear alter these corneal defences to allow infection to occur?

We have investigated the pathogenesis of

P. aeruginosa infectious keratitis with the aim of answering these questions. In this review, we summarise some of this work and its relationship to the work of other investigators in the field and outline strategies that may help fully answer the questions posed and prevent this sight-threatening disease.

BACTERIAL FACTORS AND CORNEAL INFECTION: RESEARCH WITH *P. AERUGINOSA*

Analysis of clinical isolates from cases of infectious keratitis suggests that *P. aeruginosa* has evolved a multitude of virulence factors and virulence mechanisms that allow this bacterium to survive and replicate within human corneas with resultant disease. The ability of *P. aeruginosa* to adhere to mammalian cells and tissues



Figure 1. *P. aeruginosa* infectious keratitis

or to biomaterials such as contact lenses or lens cases was the focus of many research efforts. It was intuitive that adherence in some form must be an essential aspect in the development of infection in order to allow bacterial retention in the host and the expression or action of other virulence mechanisms or factors. The goal of this research was the identification of a molecule(s) on *P. aeruginosa* that mediated attachment to specific receptors on any given surface, with the aim of blocking these interactions and preventing infection. Unfortunately, after more than two decades of research, this goal remains elusive. Several *P. aeruginosa* surface molecules including pili, flagella, outer membrane proteins and lipopolysaccharide (LPS) can modulate adherence to the cornea.^{5,6,7} Furthermore, while the glycolipid asialo-GM1 has been identified as a receptor for *P. aeruginosa* binding to mammalian cells,^{6,8} it may not be a major receptor,⁹ with *P. aeruginosa* also adhering well to other molecules on the

cornea and to many other tissues or inanimate surfaces, for example, plastic and glass.^{5,7,10,11} Additionally, any asialo-GM1 binding by *P. aeruginosa* surface molecules will activate mammalian cell signal transduction pathways,¹² which may directly or indirectly further modulate bacterial adherence. Although antibodies that inhibit *P. aeruginosa* adherence to corneal receptors can lessen the severity of corneal disease *in vivo*,^{13,14} it is not clear whether this protection is due directly to their adherence blocking properties. Overall, with multiple adhesins and potential receptors, blocking adherence remains a theoretical prevention strategy for *P. aeruginosa* infection.

Two important virulence mechanisms that have been identified recently among *P. aeruginosa* clinical isolates of infectious keratitis are the ability to invade and replicate within corneal epithelial cells (Figure 2)^{15,16} and/or the ability to kill corneal epithelial cells (bacterial cytotoxicity)

(Figure 3).¹⁷⁻¹⁹ Invasion and cytotoxicity of *P. aeruginosa* clinical isolates towards corneal epithelial cells has been demonstrated *in vitro* and *in vivo* and invasion or cytotoxicity deficient mutants of *P. aeruginosa* exhibit reduced virulence in animal models of corneal infection^{15,18} (and unpublished data). Invasion involves bacterial interaction with the CFTR (cystic fibrosis transmembrane-conductance regulator) on host cells,^{20,21} along with host tyrosine kinase activity, calcium signaling and actin cytoskeleton function.^{16,19,22} Invasion also requires an intact LPS core²³ and the flagellum protein FlhA.²⁴

Cytotoxicity is mediated by a secreted toxin called ExoU regulated by a transcriptional activator ExsA.^{18,25} Cytotoxic strains actively inhibit their uptake (invasion) by epithelial¹⁹ or host immune cells using another ExsA-regulated protein called ExoT,²⁶ which disrupts Rho-family signaling proteins that control actin cytoskeleton function.²⁷ The ExsA-regulated cytotoxicity system is a type III secretion system in which ExoU, ExoT and other co-regulated toxins are injected into host cells on contact with the bacterium.¹⁸ There is evidence of an increased prevalence of cytotoxic strains (epidemic clones) in cases of infectious keratitis over other *P. aeruginosa* infections,²⁸ which may be related to the use of contact lens disinfectants.²⁹ However, both cytotoxic and invasive *P. aeruginosa* isolates were recovered in similar numbers from cases of infectious keratitis treated at the Jules Stein Eye Institute, Los Angeles from 1998–2001 (unpublished data). Thus, although invasive strains lack the *exoU* gene needed for cytotoxicity, they are still virulent in the cornea. It can be seen how both invasion and cytotoxicity may contribute to the pathogenesis of *P. aeruginosa* infection. The ability to invade corneal epithelial cells allows bacteria to avoid many extracellular host defences such as antibodies, phagocytes, complement-mediated killing and defensins, and polar antibacterial drugs that do not readily enter cells, for example, aminoglycoside antibiotics. Alternatively, cytotoxic strains could interfere with host immune defences directly by killing host phagocytic cells. Further-

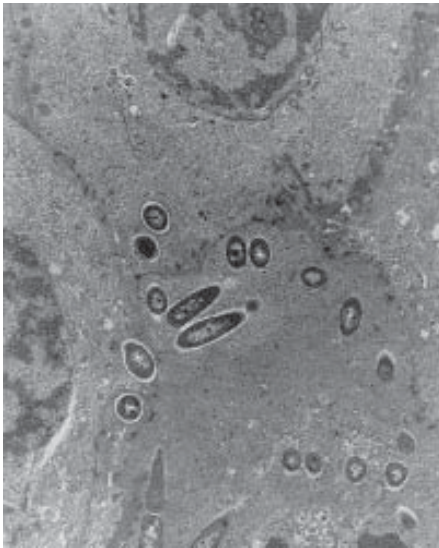


Figure 2. Electron micrograph showing invasion of corneal epithelial cells by *P. aeruginosa*

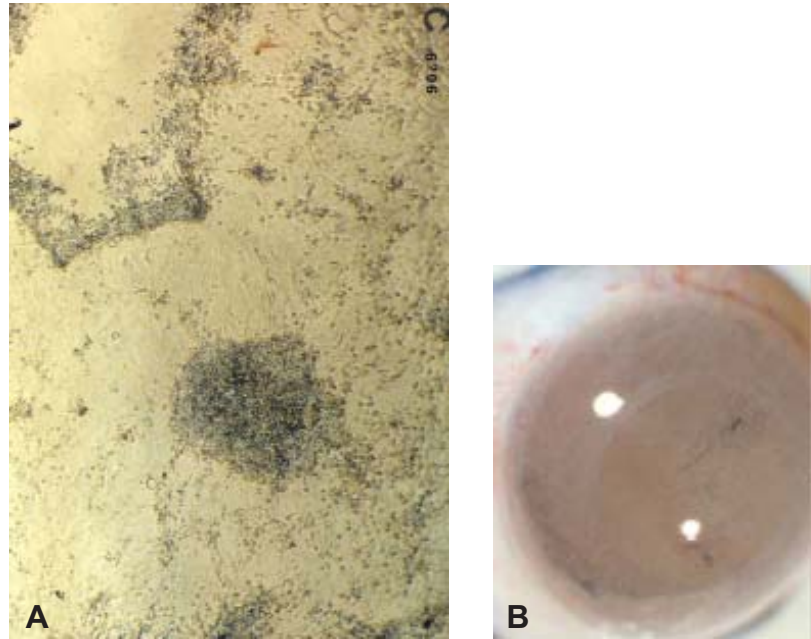


Figure 3. *P. aeruginosa* cytotoxicity (trypan blue staining) towards corneal epithelial cells in culture (A) and on the intact cornea (B)

more, the tissue damage associated with cytotoxicity is likely to increase bacterial binding and penetration of tissues and provide further nutrient availability for survival and growth.

LPS is an important virulence factor for *P. aeruginosa*, whether infecting the lungs or burn wounds or disseminating in the blood stream.^{21,23,32} LPS contributes to the pathogenesis of infectious keratitis by mediating adherence to the cornea and contact lenses^{5,7} and by mediating the internalisation and intracellular survival of invasive *P. aeruginosa* within corneal epithelial cells.^{21,23,33} Furthermore, as LPS confers resistance to complement-mediated killing, it is likely to contribute towards bacterial survival in the tear film. LPS stimulates host inflammatory and immune responses³⁴ that help to defend the eye against infection, for example, cytokine secretion, macrophage activation and defensin expression.³⁵ However, LPS-upregulated defences can also mediate

damage to the cornea and contribute to the pathogenesis of infectious keratitis. Given the multiple roles of LPS in the pathogenesis of infectious keratitis, it is perhaps not surprising that mutation of LPS to produce defects in the LPS core is one of the few manipulations that eliminates the virulence of *P. aeruginosa* in the cornea *in vivo*.^{36,37}

The contribution of other *P. aeruginosa* virulence factors to the pathogenesis of infectious keratitis has also been investigated. A rabbit model of contact lens induced microbial keratitis has been developed but is complicated by the necessity of suturing the lens-wearing eye shut to induce disease, additional to the cost and difficulty of using a sufficient number of animals for statistical analysis. Consequently, many studies have utilised the mouse 'scratched-cornea' model, in which a needle scratch to the anaesthetised corneal epithelium is needed prior to bacterial challenge to induce corneal

infection over a 24-hour period. Using this model, some established *P. aeruginosa* virulence factors—exotoxin A, elastase, alkaline protease and phospholipase C—were not necessary for corneal infection,³⁷⁻⁴⁰ while a novel virulence factor, protease IV, made a significant contribution to this ocular disease.^{38,41} Mutation of *rpoN* (a sigma factor of RNA polymerase) resulted in the complete loss of virulence for *P. aeruginosa* in the mouse cornea.³⁷ *RpoN* is needed for expression of many *P. aeruginosa* genes, including those involved in the expression of pili and flagella.⁴² Consequently, *rpoN* mutants are non-motile and exhibit significantly reduced adherence to (and invasion of) mammalian cells.^{10,11} The mouse 'scratch' model is unlikely to be fully representative of the true development of infectious keratitis in human eyes (in which infections mostly develop in the presence of soft contact lenses and an absence of needle-induced damage to the cornea). However,

the studies described and recent epidemiological studies²⁸ indicate that virulence factors for *P. aeruginosa* in the cornea may differ from some of those found important at other infection sites.

Despite advances in our understanding of the bacterial virulence factors and virulence mechanisms involved in *P. aeruginosa* keratitis, there are still many unanswered questions in this area. For example, it is still not known exactly how or when those virulence factors that have been found to be involved contribute to the pathogenesis of infectious keratitis. Furthermore, research in other areas of bacterial pathogenesis, including work with *P. aeruginosa*, has shown bacterial cells exhibit systems for sensing their environment and then signaling to each other 'quorum sensing' to alter gene expression to express a phenotype more suited for survival and replication in that environment.^{43,44} Indeed, the relative success of *P. aeruginosa* as an opportunistic pathogen may be related to its unusually high percentage of genes devoted to environmental sensing and adaptability (in addition to its intrinsic resistance to antimicrobial substances). Mutations in quorum sensing systems reduce *P. aeruginosa* ocular virulence.⁴⁵ More investigation is needed of the phenotype(s) of *P. aeruginosa* expressed in the ocular environments that allow the establishment and persistence of infectious keratitis.

WHAT ARE THE OCULAR DEFENCES AGAINST INFECTION?

Recent research suggests that ocular defences against bacterial keratitis are complex. The ocular surface environment is protected from potential infecting microbes, to which it is exposed on a constant basis, by components of both natural and acquired immunity. The potential for such immune system components to mediate significant 'collateral' damage to the corneal surface while clearing microorganisms from the eye has resulted in development/evolution of a system of immunological protection of the corneal surface that differs considerably from that found at other surfaces of the human

body. Consequently, daily corneal protection against infecting microbes relies more heavily on natural 'non-specific' immune system components to the relative exclusion/suppression of the acquired 'specific' immune system, until such time as they are needed, for example, when a pathogen establishes an infectious process.

The tear film and eyelid combination that continually washes and wipes the ocular surface represents a significant ocular defence against infection. By effectively limiting the contact time between a potential infecting microbe and the corneal surface, this 'shear-stress' based physical defence mechanism can prevent many of the microbial virulence factors and virulence mechanisms from exerting any effects on the eye. For example, invasive and cytotoxic strains of *P. aeruginosa* require two to three hours of continued interaction with target epithelial cells before they exert these effects.¹⁷ Cytotoxicity is likely to be greatly attenuated by this simple defence mechanism.^{46,47} Other *P. aeruginosa* virulence mechanisms, for example, the action of *P. aeruginosa* exotoxins, may also be attenuated. Tears could also protect the cornea from infection by inhibiting microbial growth or viability, with antimicrobial activity associated with lysozyme, lactoferrin, cationic peptides, secretory phospholipase A2, complement proteins and immunoglobulins.⁴⁸ Many potential infectious microbes may be inhibited by these various substances. However, in our laboratory, many corneal isolates of *P. aeruginosa* grew readily in samples of undiluted human tears (unpublished data), suggesting that tear film antimicrobials alone are not sufficient for prevention of *P. aeruginosa* corneal infection. However, tears do have anti-*Pseudomonas* actions that could help reduce *P. aeruginosa* virulence in healthy eyes. For example, whole tears or the mucin components of tears, aggregated *P. aeruginosa*, blocked its adherence to whole corneas and inhibited invasion and cytotoxic virulence mechanisms of this pathogen^{49,50,51} (and unpublished data). *P. aeruginosa* ocular virulence was reduced by immunoglobulins found in tears, for example, secretory IgA.^{13,14} Fur-

thermore, once bound by tear film components and in the physico-chemical environment of the tears, *P. aeruginosa* may be killed or inhibited by some tear film antimicrobials, before being physically removed from the ocular surface by tear washing and blinking. Finally, since several tear film antimicrobials are secreted in response to the presence of bacteria or their products—for example, β -defensins are produced in response to bacterial LPS³⁵—it is likely that the overall antimicrobial activity of the tears would be upregulated by the presence of bacteria in the eye.

Recent studies have shown that epithelial cells of the cornea also play a role in defence against infection. The importance of the epithelial barrier is immediately apparent in animal models of infectious keratitis, in which infection cannot be induced without bypassing the epithelial barrier via a needle scratch or other mechanism, for example, intrastromal injection. Some of the epithelial defences have begun to be elucidated and include epithelial cell polarity, secretion of cytokines, secretion of anti-microbial peptides, expression of surface-associated and secreted mucins and exfoliation of surface epithelial cells. The normal polarity of corneal epithelial cells is maintained by both membrane trafficking and tight junctions and represents a state in which proteins and other surface molecules are distributed according to function to apical (mucosal) and/or basolateral surfaces of cells. Disruption of polarity by interfering with tight junction formation, treating cells with hepatocyte growth factor or otherwise inducing cell sorting defects increases susceptibility to *P. aeruginosa* virulence mechanisms *in-vitro*.^{52,53} *In-vivo* *P. aeruginosa* appears to target cells on corneal epithelia where polarity would potentially be disrupted, for example, injured cells and cells exposed by exfoliation. Thus, polarity appears to represent a physical barrier against microbial attack, hiding unidentified basolateral surface receptor molecules that *P. aeruginosa* exploits for infection of the cornea. Epithelial derived cytokines, anti-microbial peptides and

mucins play both direct and indirect roles in defence of the cornea. Epithelial derived interleukins, IL-6 and IL-8, attract phagocytic cells to protect against microbial attack.⁵⁴ β -defensin 2, a cationic peptide secreted in response to bacterial LPS, exerts direct bactericidal activity against *P. aeruginosa* and other gram-negative bacteria.³⁵ Mucins can aggregate bacteria or block *P. aeruginosa* virulence mechanisms as described above.^{49,50} Other epithelial defences are likely to be identified but it is already clear that in combination with the tear film, the corneal epithelium is a formidable ocular defence against *P. aeruginosa* and other microbial pathogens.

HOW DO RISK FACTORS FOR INFECTIOUS KERATITIS HINDER OCULAR DEFENCES?

P. aeruginosa and other microbes associated with infectious keratitis possess significant virulence factors or mechanisms that facilitate corneal infection, yet clinically, the scarcity of corneal infection in healthy eyes in the absence of risk factors indicates that the immune defences of the cornea are successful in preventing microbial induced disease. Therefore, how do risk factors compromise host corneal immunity to allow such infections to develop? As the greatest risk factor for infectious keratitis with *P. aeruginosa* and other bacterial pathogens is the extended or overnight-wear of soft contact lenses, we will focus on how these medical devices could predispose the cornea to infection.

The integrity of the corneal defence mechanisms and overall corneal health are dependent on an appropriately oxygenated tear film regularly bathing the corneal surface. Thus, two hypotheses have dominated discussions on how a soft contact lens-wearing eye may become more vulnerable to infection:

1. contact lens induced hypoxia
2. contact lens induced stagnation/disruption of the tear film.

Both have the potential to compromise ocular defences; the role of each in the pathogenesis of infectious keratitis has not been determined.

Known effects of hypoxia on the cornea include increasing adherence of *P. aeruginosa* to corneal epithelial cells⁵⁵ and altered epithelial cell proliferation/migration rates,⁵⁶ each of which could increase *P. aeruginosa* retention on corneas of lens-wearing eyes. Other potential effects of hypoxia have not been studied but could include changes in epithelial tight junctions and cell trafficking that reduce the effectiveness of cell polarity as a defence against infection or alterations in epithelial secretion of defensins, mucins or cytokines. The advent of silicone hydrogel soft contact lenses with vastly superior oxygen permeability over conventional soft lenses should eliminate effects of hypoxia on the cornea. Clinical and experimental studies using these lenses or materials are underway to help determine the extent to which hypoxia affects corneal susceptibility to *P. aeruginosa* and other microbial infections.

When a contact lens is worn, the sweeping effect of the eyelid is likely to be markedly attenuated, significantly reducing tear exchange over the corneal surface under the lens. This would allow greater retention of microbial pathogens in the eye 'trapped' under a lens. Furthermore, because tear film-epithelial interactions are thought critical in maintaining the integrity of corneal epithelial defences against infection, 'stagnation' of the tear film may compromise epithelial polarity and defensin, mucin or cytokine expression. A recent study has shown that overnight contact lens wear alters cytokine levels in tears and consequently polymorphonuclear leukocyte (PMN) infiltration, in a manner that could affect ocular defences.⁵⁷ The lens *in situ* may also disrupt tear film composition and stability, for example, through adsorption of essential tear film components or alterations in corneal epithelial secretion or surface molecule expression. One or more of the above factors could account for contact lens-induced disruption of epithelial barrier function⁵⁸ and may hinder the antimicrobial actions of the tears. Finally, the lens itself is capable of binding bacteria or their toxins, potentially exposing the corneal surface to their deleterious effects.

It is worth noting that for *P. aeruginosa*, binding by tear film components or a lens, coupled with its inherent resistance to tear film antimicrobials, may act as a pathogenic mechanism of infectious keratitis, when stagnation of the tear film occurs under a contact lens.

In summary, many tear film and corneal epithelial defences could be disrupted by contact lens wear. The type and extent of disruption that allows infection to occur is not known. While elimination of corneal hypoxia will solve many contact lens-related complications, it remains to be seen if the risk of infections will be reduced. Given the significant role of the tear film in ocular defence, it is likely that the greatest step forward in preventing contact lens-related infectious keratitis, after eliminating hypoxia, will be the restoration of the volume and effective tear exchange in the post-lens tear film.

HOST RESPONSES AND THE PATHOGENESIS OF INFECTIOUS KERATITIS

A combination of *P. aeruginosa* virulence factors and contact lens induced compromise to host ocular defences allows the development of infectious keratitis. Once the infectious process is underway, host immune and inflammatory responses make a significant contribution to the resolution and pathology of this corneal disease. Corneal epithelial cells and other corneal tissues express LPS receptor molecules such as CD14 and Toll-like receptor 4 that bind this virulence factor, resulting in the upregulation of cytokines.³⁴ In the first instance, this clearly represents a defence mechanism that activates inflammatory and immune responses in the cornea, such as PMN infiltration and macrophage/lymphocyte activation. However, it can also be seen that the retention of LPS under a contact lens, in a stagnant post-lens tear film or in the corneal tissues could turn this defence mechanism into a significant source of tissue damage through the uncontrolled recruitment and activation of immune cells.

The role of other host factors in the pathogenesis of infectious keratitis has also

been studied. It has become clear that the availability of endogenous tissue inhibitors of metalloproteinases (TIMPS) is important in determining the level of corneal damage sustained during *P. aeruginosa* infection. Matrix metalloproteinase enzymes are capable of breaking down extracellular matrix proteins that are essential for corneal structure. Blocking the activity of TIMP-1 resulted in greater corneal damage and PMN infiltration into the cornea in an *in vivo* model of infectious keratitis and aged mice that are more susceptible to *P. aeruginosa* infection in the same model do not upregulate TIMP-1 in response to bacterial challenge.⁵⁹ There is a clear association between prolonged PMN infiltration into the cornea and corneal damage. In addition to responding to TIMPS, PMN corneal infiltration is also responsive to epithelial, macrophage or lymphocyte-derived cytokines.^{54,60-62} In studies of infectious keratitis in aged mice, corneal persistence of PMNs in response to macrophage inflammatory protein 2 (MIP-2) was associated with more severe corneal damage.⁶¹ As PMN lysosomal granules contain a diverse array of oxygen-dependent (for example, reactive oxygen species) and oxygen-independent (for example, proteinases, cationic peptides) microbial killing mechanisms that are also toxic to mammalian cells, it is understandable that PMN persistence in the cornea would be associated with an increased risk of host-induced damage. At the same time, early PMN infiltration appears essential in protecting the cornea against *P. aeruginosa* infection and later damage. Addition of exogenous IL-6 was associated with increased PMN recruitment, lower bacterial numbers in the cornea and reduced severity of *P. aeruginosa* keratitis.⁵⁴ Similarly, it is thought that part of the increased corneal damage from *P. aeruginosa* keratitis in aged mice results from delayed recruitment of PMNs into the cornea after bacterial challenge due to low cytokine levels and reduced ICAM-1 expression.^{63,64} Consequently, greater numbers of *P. aeruginosa* survive in the cornea at later time points,^{61,64} contributing to a more severe keratitis through the greater expression of the many *P. aeruginosa* virulence

factors described earlier. In addition, once *P. aeruginosa* gains sufficient numbers, PMNs that later infiltrate and persist in the cornea⁶¹ may be unable to phagocytose bacteria due to ExxA-regulated inhibition, cytotoxicity or other factors (see above), causing release of lysosomal granule contents that result in host damage to the cornea. Also, it has been shown that proteinases released from PMNs are activated by *P. aeruginosa* virulence factors, for example, exotoxin A, elastase and alkaline protease, providing another possible mechanism of host-mediated damage resulting from *P. aeruginosa* infection.⁶⁵

Exciting progress has been made in our understanding of the host responses to *P. aeruginosa* corneal infection once the disease process has begun. This knowledge is likely to contribute to the development of new therapeutic strategies that lessen the severity of host-mediated corneal damage in infectious keratitis. However, it is unclear which host defences normally protect the cornea from initiation of bacterial infection and at what stage after initiation these host immune responses begin to become detrimental rather than beneficial to the outcome of infection.

CONCLUSION: FUTURE RESEARCH DIRECTIONS

Solid progress has been made in our understanding of the pathogenesis of infectious keratitis with much work focused on the opportunistic bacterial pathogen *Pseudomonas aeruginosa*. Unfortunately, none of the questions asked at the introduction of this manuscript has yet been fully answered. Most of the research conducted to date has focused on the events that occur once an infection is established. We do not know which bacterial phenotype(s) allows corneal infection to occur, how the corneal surface is normally so resistant to bacterial infection, how contact lens wear or other risk factors compromise these ocular defences, or the extent to which corneal pathology is mediated by host or bacterial factors. In our opinion, two essential factors are needed to answer these questions and understand the pathogenesis of bacterial keratitis. They are:

1. The successful development of a contact lens-wearing animal model of this infection that does not require scratching the eye or suturing the eyelid to induce disease; current models are either independent of hypoxia, tear film and epithelial interactions (mouse 'scratch' model) or unlikely to be representative of the initiation of contact lens-induced keratitis (rabbit 'sutured eye' model).
2. Understanding the key mechanism(s) that render the healthy human corneal surface so resistant to infection.

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Author's address:

Suzanne M J Fleiszig
School of Optometry
688 Minor Hall
University of California at Berkeley
Berkeley, CA 94720-2020
USA