The Impact of Inoculation Parameters on the Pathogenesis of Contact Lens–Related Infectious Keratitis

Connie Tam, James J. Mun, David J. Evans, and Suzanne M. J. Fleiszig

PURPOSE. Contact lens wear predisposes to Pseudomonas aeruginosa keratitis, but the mechanisms involved remain unclear. An in vivo model was used to study lens inoculation conditions enabling disease.

METHODS. Custom-made hydrogel contact lenses were fitted to rats after incubation in P. aeruginosa approximately 10^{11} cfu/mL (3 hours) or approximately 10^{3} cfu/mL (24 hours). Another group was inadvertently inoculated with a suction pen previously used with high inocula, but rinsed in ethanol and stored dry (6 months). Some corneas were tissue paper-blotted to cause fluorescein staining before lens fitting. Contralateral eyes were untreated. Twenty-four hours after disease detection, lenses were transferred to naïve rats or examined by confocal microscopy before homogenization to quantify viable bacteria. After lens removal, corneas were washed to collect nonadherent bacteria and were analyzed by immunohistochemistry.

RESULTS. All eyes challenged with unworn contaminated lenses developed keratitis after approximately 7 to 10 days. Disease delay and severity were unaffected by inoculum parameters or tissue blotting but occurred sooner with lenses transferred from infected eyes (~2 days). Worn lenses and corneal washes contained infecting bacteria. Posterior, not anterior, lens surfaces harbored P. aeruginosa biofilms that penetrated the lens matrix. Diseased corneas showed an infiltration of phagocytes and T-lymphocytes.

CONCLUSIONS. P. aeruginosa induces keratitis in this lens-wearing model after a single inoculation. Delayed disease onset was interesting considering the greater keratitis risk during extended wear. Infection did not require the disruption of corneal barrier function before lens wear and occurred without exposure to lens care solutions. The data suggest that keratitis involves biofilm formation or other bacterial adaptations in vivo. (Invest Ophthalmol Vis Sci. 2010;51:3100–3106) DOI: 10.1167/iovs.09-4593

Contact lens wear is the most common predisposing factor for Pseudomonas aeruginosa. However, the mechanisms by which contact lens wear predisposes the otherwise resistant cornea to infection by this or any other pathogen are not well understood. Importantly, the availability of daily disposable contact lenses, and of lens materials that are hyperpermeable to oxygen, has not eliminated the risk for contact lens–related infection. Thus, factors other than lens deposits and hypoxia appear to be involved in pathogenesis of this disease.

Our understanding of the factors that initiate contact lens–related infectious keratitis has been limited, in part, by the difficulty of obtaining lenses that fit the eyes of rodents. Although researchers have studied lens wear in rabbits and guinea pigs, these animals are expensive and awkward to work with. Rabbit models involve surgical removal of nictitating membranes, or suture-mediated eyelid closure, to overcome lens dislodgement and to maximize lens effects such as hypoxia. Guinea pig models (lens-wearing guinea pigs without surgical interventions) are of value for studying the ocular safety of lenses and the effects of extended lens wear on corneal physiology, but these animals are not susceptible to P. aeruginosa infection. Accordingly, most of our knowledge to date about how P. aeruginosa and other microbes infect the cornea has necessarily been derived from experiments using cultured corneal epithelial cells or in vivo rodent models in which lenses are not used. To enable susceptibility to infection without contact lens wear, researchers have heavily used a scarification model, or they have performed intrastromal injection of the infectious agent (for reviews see Refs. 8–10). Although these models are useful tools for elucidating the host responses to an already established infection or for exploring the role(s) of bacterial virulence factors in maintaining persistence within an infected cornea, they are of limited value for studying how lens wear enables disease susceptibility or for elucidating factors involved in the initiation of disease in the case of lens-related infection.

A rat model of extended hydrogel lens wear has been published. Experiments using this model have shown the induction of P. aeruginosa keratitis, the upregulation of proinflammatory cytokines, and dendritic cell and neutrophil infiltration of the cornea. The model has also been used to show increased proinflammatory responses and increased susceptibility to P. aeruginosa infection for low- versus high-Dk lenses. Thus, this model has advanced our understanding about P. aeruginosa infection once it has been initiated and about lens parameters that contribute to risk. Because the method requires repeated bacterial inoculation to enable infection, and consequently confusion in the timing of challenge, it is less useful for studying initiating events related to the bacteria or the early corneal response to them.

The purpose of the present study was to determine whether the previously published rodent model could be modified to enable P. aeruginosa keratitis using a single inoculation event (synchronizing the inoculum) so that conditions leading to disease susceptibility/initiation could be better defined. We

From the 1School of Optometry, the 2Vision Science Program, and the Graduate Groups in 3Microbiology and 5Infectious Diseases and Immunity, University of California, Berkeley, California; and the 4College of Pharmacy, Touro University-California, Vallejo, California.

Supported by National Eye Institute Grant R01-EY011221 (SMJF).

Submitted for publication September 4, 2009; revised November 10 and December 15, 2009; accepted January 24, 2010.

Disclosure: C. Tam, None; J.J. Mun, None; D.J. Evans, None; S.M.J. Fleiszig, None

Corresponding author: Suzanne M. J. Fleiszig, School of Optometry, University of California, 688 Minor Hall, Berkeley, CA 94720-2020; fleiszig@berkeley.edu.
hypothesized that the adjustment of bacterial growth conditions and the disruption of host epithelial barrier function (to fluorescein) by tissue paper blotting of the cornea might allow *P. aeruginosa* keratitis to develop after a single inoculation if given sufficient time. Surprisingly, results revealed that disease occurred under all conditions tested with a significant delay that was reduced only when the inoculum vehicle was a lens transferred from an already infected eye. Taken together, the data suggest that bacterial adaptation to the in vivo environment, possibly enabled by biofilm formation found on the posterior surface of lenses removed from infected eyes, is involved in the pathogenesis of contact lens–related keratitis.

**MATERIALS AND METHODS**

**Rat Contact Lenses**

Low-Dk hydrogel contact lenses (69% water, Dk 26) were custom made to fit rat corneas and had the following dimensions: 5.34-mm diameter, 2.4-mm base curve, 80-μm center thickness, and 40-μm edge thickness. Integrity of the lenses was inspected under a stereomicroscope to ensure edges were smooth before lens fitting.

**Bacteria Strain and Inoculum Preparation**

*P. aeruginosa* invasive strain PAO1 expressing GFP on a pSMC2 plasmid (PAO1-GFP) was used in all experiments. Bacteria were grown on tryptic soy agar (TSA) plates supplemented with carbenicillin 300 μg/mL at 37°C for approximately 16 hours. For high-inoculum conditions, bacteria were suspended in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St. Louis, MO) to a concentration of 10^{11} cfu/mL. For low-inoculum conditions, a single colony of bacteria was picked by a sterile toothpick and inoculated into 3.5 mL modified Mian’s medium (7.5 mM NaH_{2}PO_{4}, 16.8 mM K_{2}HPO_{4}, 10 mM MgSO_{4}, 0.2% NaNO_{3}, 10 mM CH_{3}COONa) contained within a contact lens case resulting in an initial concentration of 10^{3} cfu/mL and reaching a final concentration of 10^{7} cfu/mL after 24 hours at room temperature. Bacterial counts in the inocula were confirmed by viable counts. Contact lenses were incubated with either a high inoculum for 3 hours or a low inoculum for 24 hours at room temperature before placement on the left cornea of each rat. Lenses were not rinsed to remove nonadherent bacteria before insertion (~10 μL soaking inoculum was carried over with the lens). Just before inoculation there were approximately 10^{9} cfu adherent *P. aeruginosa* on each lens after incubation under high-inoculum conditions and approximately 10^{6} cfu on each lens under low-inoculum conditions.

**Lens-Wearing In Vivo Model**

Three-month-old female Lewis rats were purchased from Charles River Laboratories Inc. (Wilmington, MA). During the experiment, animals were housed individually and were allowed normal activity without behavior restraints in a controlled environment (temperature at 72°F ± 4°F, humidity at 50%–90%, and 12-hour light/12-hour dark cycles). Before insertion and removal of inoculated contact lenses or examination and image-capturing of eyes, animals were subject to light anesthesia by administration of 2.5% isoflurane in medical-grade oxygen using a precision vaporizer. Corneas of some animals were blotted once with 1-psy tissue paper (Kimwipe; Kimberly-Clark, Irving, TX) before fitting with high-inoculum-soaked lenses. The blotting procedure sufficiently disrupted the corneal epithelium to allow extensive fluorescein staining (Fig. 1). In other experiments, bacteria were inadvertently introduced into the lens-wearing rat eye during handling with a previously “disinfected” suction pen. The pen had been used 6 months previously for high-inoculum experiments, ethanol-rinsed, air-dried, and stored dry for 6 months before use for fitting lenses that were otherwise sterile.

Each experimental group consisted of at least three animals. Contraocular eyes served as controls and were not fitted with lenses or inoculated with bacteria. Animals were monitored daily for corneal pathology. Twenty-four hours after a faint opacity was observed on the cornea, images were collected using a three-chip cooled camera (Optronics, Goleta, CA) attached to a stereomicroscope (Stemi 2000-C; Carl Zeiss, Thornwood, NY). A 5-point extended grading system (0–4) that assesses four characteristics of the pathology was used as described previously. This involved scoring the area of the opacity, density of the central and peripheral opacities, and epithelial surface quality. The calculated sum of scores for these four characteristics ranges from 0 (no infection) to a maximum of 16 (severe infection). When lens dislodgment occurred before disease initiation, the lens was not reinserted, but animals were observed for the remainder of the study.

After the development of corneal pathology, lenses were removed, and infected corneas were immediately washed with 10 μL PBS to collect nonadherent bacteria for quantification. Worn lenses of diseased animals were examined by laser scanning confocal microscopy before homogenization in 100 μL PBS. In some experiments, these lenses (from the infected low-inoculum group) were transferred to naive rats. Viable bacteria in ocular washes and on worn lenses were quantified by serial dilution and subsequent plating on nonselective TSA, with selective media inoculated in parallel: 300 μg/mL carbenicillin-supplemented TSA to select plasmid-bearing bacteria, MacKonkey agar to select Gram-negative bacteria, and Cetrimide agar to select *P. aeruginosa*. Animals were killed by inhalation of 5% isoflurane before enucleation of eyes, which were processed for frozen-sectioning and immunofluorescence microscopy. All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care and Use Committee of the University of California, Berkeley.

**Immunohistochemistry of Corneal Tissue**

Whole eyes were fixed in 4% paraformaldehyde (wt/vol) for 1 hour at room temperature, infiltrated with 30% sucrose in PBS (wt/vol) overnight at 4°C, and frozen in optimal cutting temperature embedding medium before sectioning at 5- to 8-μm thickness with a cryostat (CM1900; Leica Microsystems, Bannockburn, IL). To block nonspecific staining, sections were incubated in PBS containing 3% bovine serum albumin (BSA), 3% goat serum, and 0.1% Triton X-100 for 1 hour at room temperature. Sections were incubated in mouse monoclonal antibody raised against rat CD11b/c (labels the CR3 complement receptor found on most professional phagocytes), ED-1 (a monocyte/T-cell receptor or CD4; BD Pharmingen, San Jose, CA; 1:100 dilution in blocking buffer) overnight at 4°C and then were washed three times for 5 minutes each in PBST (0.1% BSA and 0.1% Triton X-100 in PBS). Rhodamine-Red–conjugated goat antimouse IgG's secondary antibody (Invitrogen, Carlsbad, CA; 1:5000 dilution in blocking buffer) was used for 1 hour in room temperature,
followed by four washes for 10 minutes each in PBS. Samples were mounted and counterstained with mounting medium containing DAPI (Vectorshied; Vector Laboratories, Burlingame, CA) to label cell nuclei (blue) and were viewed under an epifluorescence microscope (IX70; Olympus, Center Valley, PA).

**Laser Scanning Confocal Microscopy of Contact Lenses**

Contact lenses removed from infected animals were imaged with an upright confocal system (LSM 510; Carl Zeiss GmbH, Jena, Germany) using the 488-nm laser line to study bacterial binding as represented by the GFP signal. To enable viewing, contact lenses were cut in half, moistened with PBS, and flattened on glass slides. To enable both the anterior and the posterior sides of each lens to be studied and to avoid possible signal detection differences along the z-depth of thick samples, the lens halves were oriented in opposite directions so that both the posterior and the anterior surfaces were facing the objective and could be viewed in the same plane. Stacks of horizontal-plane (x-y) images from three random fields were acquired at 1-μm intervals down the z direction until signals diminished. Image projections and vertical cross-sections (x-z and y-z planes) were generated (LSM Image Browser; Zeiss). Pilot studies confirmed that bacteria attached to contact lenses removed from infected corneas retained the GFP plasmid.

**Statistical Analysis**

Numerical data are expressed as median values with lower and upper quartiles (Q1:Q3). The nonparametric Mann-Whitney U test was used to compare differences between two groups and the Kruskal-Wallis test for three or more groups. P < 0.05 was considered statistically significant.

**RESULTS**

**Effect of Inoculum Parameters on Contact Lens–Induced P. aeruginosa Keratitis**

All contact lens–wearing rat eyes developed corneal opacity after a single inoculation with *P. aeruginosa* at the time of lens fitting, irrespective of inoculum conditions or corneal blotting. In each instance, initiation of disease was delayed (Fig. 2A). There was no significant difference in the median delay in disease onset between the low-dose inoculation group (8 days) and the high-dose group (7 days) (*P* = 0.617; Mann-Whitney U test). Inadvertent inoculation during lens handling with a previously “sterilized” suction pen (see Materials and Methods) also enabled disease with a comparable onset delay (10 days) that was not significantly different from that of low- and high-dose inocula (*P* = 0.240; Kruskal-Wallis test). Transferred lenses from previously infected eyes showed a trend toward a shorter delay in keratitis onset (median 2 days), which was statistically significant compared to the inadvertent inoculum group (*P* = 0.048; Mann-Whitney U test) (Fig. 2A). Blotting of rat corneas with tissue paper before inoculation (high dose) and lens fitting to allow fluorescein staining (Fig. 1) did not impact the timing of disease onset (median, 8.5 days) (*P* = 1.000; Mann-Whitney U test compared with a high-dose inoculum without blotting).

Disease severity scores (Fig. 2B) ranged from a median of 6 (transferred lens) to 9 (high inoculum) out of a maximum of 16. There was no significant difference in disease severity scores among the low-, high-, and inadvertent inoculum groups (*P* = 0.427; Kruskal-Wallis test) or between the blotted and unblotted lens-wearing eyes at high inocula (*P* = 0.53; Mann-Whitney U test). None of the untreated contralateral eyes or eyes in a control group wearing sterile lenses for up to 14 days showed corneal disease.

As shown in Figures 3A and 3B, ocular washes and lens homogenates from infected eyes revealed surprisingly consistent numbers of bacteria in each location (~1.5–2.4 × 10⁵ cfu and ~0.57–1.3 × 10⁶ cfu, respectively, for all animals). For both parameters, there was no significant difference among any of the groups (*P* = 0.659, *P* = 0.688, respectively).

Selective media were used in control experiments to verify that bacteria recovered from infected eyes were from the inoculum introduced at the time of lens insertion. This was done by simultaneously plating ocular surface washes and homogenates of lenses removed from infected eyes on both nonselective TSA and selective media (see Materials and Methods). Similar colony numbers were recovered on these media for lens homogenates and surface washes (data not shown). Further, only one colony type was noted growing on TSA plates. These results confirmed retention of the GFP-plasmid in the lens-wearing eye in vivo and recovery of the inoculated bacteria with no significant colonization by any other type of recoverable bacterium. These data are consistent with our previous study showing the retention of a similar plasmid by *P. aeruginosa* in an infected murine cornea in vivo.¹⁵

**Gross Pathology and Microscopy of Diseased Corneas**

Cross-sections of diseased and control corneas were examined macroscopically and using phase-contrast and fluorescence mi-
croscopy (Fig. 4). Two examples of eyes with severe disease (clinical scores 10 or greater) are shown in Figure 4A. In each instance, there was a large central corneal opacity (top panels, one showing the lens in situ), gross disruption of all layers of the cornea with significant epithelial swelling (middle panels), and extensive cellular infiltration (blue cells, solid arrows). Notably, there was significant penetration of GFP-expressing \textit{P. aeruginosa} (green, dashed arrows) in each infected cornea (lower panels). An example of eyes with less severe pathology (clinical scores lower than 10) is shown in Figure 4B. A large central corneal opacity was observed (top panel, with lens in situ) but was fainter than that observed with severely diseased eyes. There was a similar disruption of all corneal layers, corneal epithelial swelling, and cellular infiltrate (middle and bottom panels). However, GFP-expressing bacteria were not observed in corneas with mild disease (bottom panel), suggesting that bacteria had not entered the cornea or that they were cleared from the cornea at the time of kill.

Figure 5 shows an example for a rat eye that had developed moderate \textit{P. aeruginosa} keratitis after 5 days of inoculated lens wear. Immunostaining of corneal cross-sections showed infiltrates of cells that labeled with monoclonal antibodies reactive with CD11b/c (CR3 complement receptor), ED-1 (a monocyte/macrophage marker), the αβ T-cell receptor, and CD4. Although not shown, a similar pattern of immunolabeling was observed with severely diseased corneas at other times after inoculation. In all instances, eyes with lens-associated \textit{P. aeruginosa} keratitis showed an infiltrate of CD11b/c/H11001 cells, suggestive of professional phagocytes (e.g., PMNs and macrophages).

\textit{P. aeruginosa} Biofilm Formation on and within Contact Lenses Worn In Vivo

Laser scanning confocal microscopy of lenses removed from rat eyes with \textit{P. aeruginosa} keratitis showed classical bacterial biofilm structures across the posterior lens surface (15- to 20-\textmu m total thickness) (Figs. 6B, 6D). These biofilms were partially embedded within the lens (\textless 10–15 \textmu m into lenses of approximately 80 \textmu m total thickness), with the remaining (\textless 5 \textmu m) of biofilm exposed on the lens surface. In contrast, the anterior surface of worn lenses showed only isolated bacteria embedded up to \textless 10 \textmu m beneath the surface (Fig. 6A, anterior surface of lens shown in Fig. 6B). Inoculated lenses, \texttimes 10^3 cfu/mL PAO1-GFP for 24 hours (low inoculum; Fig. 6C) or \texttimes 10^{11} cfu/mL for 3 hours (high inoculum; not shown) did not show biofilm structures before fitting.

DISCUSSION

The data presented in this report show that a single inoculum of \textit{P. aeruginosa} introduced with a hydrogel contact lens can enable keratitis in rats over a period of 2 to 14 days of wear.
Bacteria derived from the original inoculum were found on the lens and at the ocular surface (under the lens) in all diseased eyes. The bacteria were also found within some, but not all, of the diseased corneas. Pathology was associated with infiltration of professional phagocytes (CD11b/c+ and ED-1+ cells) and T-lymphocytes including CD4+ cells. Neither the delay in disease onset nor disease severity was significantly influenced by inoculum parameters (inoculum size or type) or the state of the epithelial barrier (to fluorescein). The delay was, however, significantly reduced when lenses were transferred from diseased eyes to naive animals. In all cases, there was extensive P. aeruginosa biofilms on, and embedded within, the posterior, but not the anterior, lens surfaces.

P. aeruginosa has a large genome with an unusual number of genes devoted to environmental adaptation. These include more than 70 two-component sensory-regulatory systems that enable it to adjust to a diverse array of environmental conditions. Some of these regulatory systems have been shown to impact virulence factor expression and corneal disease pathogenesis in vivo. The noted delay in disease onset, and the reduced delay when lenses were transferred from infected eyes to naïve animals, suggests that pathogenesis of contact lens–related keratitis could involve bacterial adaptation to the in vivo environment, with accompanying changes to gene expression to enable a virulent bacterial phenotype/genotype. Also supporting bacterial adaptation to factors found in vivo are our (unpublished, 2009) data showing that P. aeruginosa can acquire an enhanced capacity to penetrate multilayered corneal epithelium on repeated exposure to epithelial cells. If adaptation does occur, and if this is critical to disease pathogenesis, then studies of the mechanisms involved (e.g., aspects of the in vivo environment that trigger these changes and the profile of bacterial genes impacted) could point toward new targets for disease prevention.

The biofilm formation found on posterior lens surfaces could also be involved in pathogenesis. That involvement could be direct, or indirect by enabling bacteria to persist in the eye so that there can be adaptation to the in vivo environment. Existence within a biofilm on the posterior surface would protect bacteria from the physical removal forces of blinking and tear flow. Further, it is well known that biofilm formation protects P. aeruginosa and other bacteria from killing by antimicrobial substances and host immune defenses while providing a niche within which bacteria can alter gene expression and transfer antibacterial resistance genes. Bacterial growth as biofilms on contact lenses in vitro has been shown to enhance resistance to contact lens disinfectants and to phagocytic cells. Thus, biofilm growth on the posterior lens surface in vivo could protect bacteria against both epithelial cell and tear-derived antimicrobial factors, such as complement, IgA, and defensin antimicrobial peptides. Further studies will be needed to delineate the relative contributions of biofilm and other in vivo adaptations in the initiation of contact lens–related keratitis.

Interestingly, in one set of experiments, eyes that were not deliberately inoculated became infected during lens wear. GFP-expressing P. aeruginosa that grew on all the selective media were isolated from the lens and eye washes of these infected eyes. The source of the bacterial inoculum was traced back to the suction pen used for inserting into eyes; the pen had been...
Contact Lens Inoculation and *P. aeruginosa* Keratitis

In conclusion, contact lens–wearing animal models of microbial keratitis provide significant advantages over established models involving scarification or intrastromal injection by providing a clinically relevant means to study the combination of microbial and host factors that predispose to infection. The data shown in this study suggest that bacterial adaptation to the contact lens–wearing cornea, possibly involving the biofilm formation found on the posterior surfaces of lenses, contribute to the pathogenesis of this disease.

Until recently, research in this field had been predominantly patient or cell culture based, which has limited the approaches used to either US Food and Drug Administration-approved methods or to in vitro experiments. The result of those significant (and costly) efforts has been the development of products that have had little impact on the incidence of the sight-threatening complications of contact lens wear. Continued availability of appropriately manufactured contact lenses that fit animals will be critical for developing an effective means to prevent these iatrogenic and potentially blinding conditions.

**Acknowledgments**

The authors thank Gerald B. Pier (Harvard Medical School, Boston, MA) for providing the pSMC2 plasmid and Steven Ruzin and Denise Schichnes (Biological Imaging Facility, University of California, Berkeley) for use of the confocal microscopy system and for expert technical assistance.

**References**


