Pseudomonas aeruginosa Infectious Keratitis in a High Oxygen Transmissible Rigid Contact Lens Rabbit Model

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PURPOSE. To establish a rabbit model of infectious *Pseudomonas aeruginosa* keratitis using ultrahigh oxygen transmissible rigid lenses and characterize the frequency and severity of infection when compared to a non-oxygen transmissible lens material.

METHODS. Rabbits were fit with rigid lenses composed of ultrahigh and non-oxygen transmissible materials. Prior to wear, lenses were inoculated with an invasive corneal isolate of *P. aeruginosa* stably conjugated to green fluorescent protein (GFP). Corneas were examined before and after lens wear using a modified Heidelberg Rostock Tomograph in vivo confocal microscope. Viable bacteria adherent to unworn and worn lenses were assessed by standard plate counts. The presence of *P. aeruginosa*-GFP and myeloperoxidase-labeled neutrophils in infected corneal tissue was evaluated using laser scanning confocal microscopy.

RESULTS. The frequency and severity of infectious keratitis was significantly greater with inoculated ultrahigh oxygen transmissible lenses. Infection severity was associated with increasing neutrophil infiltration and in severe cases, corneal melting. In vivo confocal microscopic analysis of control corneas following lens wear confirmed that hypoxic lens wear was associated with mechanical surface damage, whereas no ocular surface damage was evident in the high-oxygen lens group.

CONCLUSIONS. These data indicate that in the absence of adequate tear clearance, the presence of *P. aeruginosa* trapped under the lens overrides the protective effects of oxygen on surface epithelial cells. These findings also suggest that alternative pathophysiological mechanisms exist whereby changes under the lens in the absence of frank hypoxic damage result in *P. aeruginosa* infection in the otherwise healthy corneal epithelium.

Keywords: cornea, epithelium, contact lens, hypoxia, *Pseudomonas aeruginosa*

Microbial keratitis (MK) is the most devastating complication associated with contact lens wear and can result in substantial loss of vision, and in severe cases, blindness. For more than three decades, *Pseudomonas aeruginosa* (PA) has been repeatedly identified as the most frequently isolated pathogen in all culture-positive cases of contact lens-related microbial infection of the cornea.^{1–6} Previous clinical and basic studies have focused on the role of hypoxic damage to the surface epithelium and increased microbial adhesion as a key event in initiating infection.^{7–10} The contributory role of hypoxia has been established by studies demonstrating a direct relationship between *P. aeruginosa* binding to corneal epithelial cells in response to reduced lens-oxygen transmissibility and a corresponding increase in lipid raft-mediated *P. aeruginosa* internalization in corneal epithelial cells.^{11–16} In concert with this data, two prior laboratories have used low oxygen transmissible hydrogel lenses inoculated with *P. aeruginosa* to initiate infection in rodent models.^{17,18}

While epithelial surface damage resulting from low oxygen transmissible lens wear may promote bacterial adhesion to the corneal surface in vivo, exposure of the human cornea to anoxia using nitrogen goggles alters epithelial desquamation but does not increase *P. aeruginosa* adherence to exfoliated epithelial cells.¹⁹ Related studies using filter paper injury to mimic epithelial surface damage from lens wear in the mouse eye in the absence of a hypoxic stimulus followed by direct

inoculation with *P. aeruginosa* have also found that the cornea fails to infect from surface damage alone.²⁰ Moreover, low oxygen transmissible lens wear, in the presence of a preexisting epithelial defect, fails to alter the time course of *P. aeruginosa* infection in the rat eye.¹⁷ Taken together, these studies suggest that either corneal epithelial surface damage alone or that due to hypoxia is not sufficient to produce *P. aeruginosa* infection of the otherwise healthy corneal epithelium and that the presence of a lens is required. Given the critical role of the lens in the pathogenesis of infection, it is not surprising that current epidemiological evidence indicates that the use of high oxygen transmissible lenses, which eliminate hypoxia-induced corneal surface damage, has failed to alter the overall incidence of microbial infection associated with contact lens wear.²¹

To date, our primary limitations in assessing the role of contact lenses in the pathobiology of infection include the multifactorial nature of the disease and the lack of a standard animal model that incorporates the use of contemporary high-oxygen lens materials. Previous work in our laboratory has identified a role for inflammation in mediating *P. aeruginosa* colonization of contact lens surfaces.^{22,23} We have further shown that the accumulation of dying neutrophils under the lens promotes bacterial uptake into the corneal epithelium of the lens-wearing rabbit eye.²² In the present study, we demonstrate that in the absence of adequate tear clearance, a rigid lens inoculated with *P. aeruginosa* consistently induced

severe corneal infection in the rabbit model during wear of both non-oxygen transmissible (anoxic) and ultrahigh oxygen transmissible polymers. We further show that, in contrast to all prior studies of contact lens-related hypoxia with non-bacterial-laden lenses, infection was greatest with wear of colonized ultrahigh oxygen transmissible lenses. Thus, this provides a novel clinically relevant model of disease to investigate both the host response to *P. aeruginosa* and the ability of *P. aeruginosa* to penetrate, invade, and traverse the normally resistant corneal epithelium.

MATERIALS AND METHODS

Animals

Eighteen female New Zealand White rabbits (2.5–3.5 kg body weight; Charles River Laboratories, Wilmington, MA, USA) were used. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Procedures were performed as approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee. Prior to lens fitting, all rabbits underwent a partial nictitating membranectomy to facilitate lens retention. Membranectomies were performed under anesthesia with an intramuscular injection of 30 mg/kg ketamine HCL (Ketaset; Ft. Dodge Animal Health, Ft. Dodge, IA, USA) and 1.5 mg/kg xylazine (Anased; Lloyd, Inc., Shenandoah, IA, USA) followed by one drop of topical proparacaine HCL 0.5% (Alcon Laboratories, Fort Worth, TX, USA). After a 1-week recovery period, rabbit corneas were scanned at baseline using in vivo confocal microscopy. There was a 3-day washout period between confocal scanning and lens fitting. Following lens removal and in vivo confocal microscopy, animals were euthanized under anesthesia with an intravenous injection of 120 mg/kg pentobarbital sodium (Euthasol; Virbac Animal Health, Fort Worth, TX, USA).

Bacteria Strain

Frozen stocks of *P. aeruginosa* strain 6487, an invasive corneal isolate that is stably conjugated to a green fluorescent protein (GFP)-expressing plasmid (pSMC2) with a carbenicillin-resistant cassette (gift from Suzanne Fleiszig, UC Berkeley, Berkeley, CA, USA), were stored at -80°C in tryptic soy broth (Sigma-Aldrich Corp., St. Louis, MO, USA) containing 10% glycerol. Bacteria were grown overnight at 37°C on a master plate consisting of tryptic soy agar (Sigma-Aldrich Corp.) supplemented with carbenicillin (300 $\mu\text{g/mL}$). Prior to each experiment, a single colony was selected and grown on Mueller Hinton agar (Sigma-Aldrich Corp.) slants overnight for approximately 16 hours at 37°C . Bacteria were resuspended with sterile PBS and adjusted to 0.100 optical density (OD) in a spectrophotometer (SmartSpec SpecPlus; BioRad, Hercules, CA, USA) at 650 nm (approximate concentration of 1×10^8 CFU/mL) for contact lens inoculation.

Contact Lenses

Rigid contact lenses composed of either PMMA (non-oxygen transmissible, diffusivity solubility [Dk] 0) or tisilfocon A (TA; high-oxygen permeable, Dk 160) with identical parameters (diameter, 14.0 mm; base curves, 7.80–8.20 mm; Menicon, Nagoya, Japan). Lenses were inoculated on the concave side only with 200 μL of a suspension containing 10^7 CFU *P. aeruginosa* in PBS and incubated overnight (approximately 18 hours) at 37°C . Lenses were rinsed with PBS to remove all nonadherent bacteria prior to insertion. For contact lens fitting, rabbits were administered an intramuscular injection of

30 mg/kg ketamine and 1.5 mg/kg xylazine. Optimal lens fitting was confirmed using sodium fluorescein (FUL-GLO Fluorescein sodium strips; Akorn, Lake Forest, IL, USA) and a handheld biomicroscope with a cobalt blue filter. In the first set of experiments, rabbits were fit with an inoculated lens in the right eye only and followed for signs of disease. Drying on the outer surface of the rigid lens prevented early assessment of a faint corneal opacity without placing the rabbit under additional anesthesia to move the lens and examine the cornea. Thus, animals wore the lens until they presented with clinical signs of visible redness and accompanying mucopurulent discharge, which was then scored as the outcome measure for clinical onset of disease. In the second set of experiments, performed approximately 4 months later by a different trained laboratory technician, rabbits were fit with an inoculated lens on the right eye and a sterile control lens on the left eye and followed over 3 days. Following lens wear, lenses were cleaned with a solution of 50% bleach in PBS in a 50 mL conical tube for 30 minutes with agitation, followed by washing in sterile water three times for 10 minutes each. Lenses were then washed in 70% ethanol solution for 30 minutes with agitation and again washed with sterile water four times for 5 minutes each. Lenses were allowed to air dry in the cell culture hood overnight.

In Vivo Confocal Microscopy

In vivo confocal microscopy (IVCM) was performed using a modified Heidelberg Rostock Tomograph confocal microscope with a Rostock Corneal Module (Heidelberg, Germany).^{24,25} Prior to scanning, rabbits were anesthetized with an intramuscular injection of 50 mg/kg ketamine and 5 mg/kg xylazine. One drop of topical proparacaine HCL 0.5% (Alcon Laboratories) was instilled into the eye being scanned. Lubrication was maintained on the contralateral eye using a preservative-free artificial tear (Allergan, Irvine, CA). A custom-designed polished PMMA washer (1.2-cm outer diameter, 2-mm inner diameter, 200- μm thick) was placed on the Tomocap (Heidelberg, Germany) to eliminate the reflections that can interfere with superficial epithelial imaging.^{24,26} Genteal Gel (Alcon Laboratories) was used to couple the applanating tip to the cornea. Image acquisition was performed as previously described.²⁴ Corneas were scanned at a lens speed of 60 μm per second. A minimum of three scans were performed on each cornea. While no apparent corneal epithelial surface damage from the use of topical proparacaine was evident during scanning, all rabbits underwent a 3-day recovery period prior to inoculation.

Determination of Viable Bacteria on Contact Lens Surfaces

To establish the level of inoculum adherent to each lens material and to confirm the inoculum load for each round of lens fittings, lenses were prepared in parallel to the lens being fitted. For nonworn lenses, viable bacteria adherent to the lens surface were quantified by standard plate counts. Lenses were placed in 5 mL PBS and vortexed on high for 2 minutes.²⁷ Vortexed samples were then serially diluted in PBS, plated in triplicate on Mueller Hinton agar plates, and cultured overnight at 37°C . For worn lenses, after removal lenses were placed in 1.5 mL PBS for subsequent vortexing to disperse adherent bacteria and plated in triplicate. Infected eyes were also washed with 1 mL PBS to collect *P. aeruginosa* from the ocular surface. For eye washes, 1 mL of PBS was instilled into the lateral canthus using a 1-mL pipette. Using a transfer pipette, eyewash was collected by continuous removal from the medial

TABLE 1. Disease Pathology Grading Scale

	0	1	2	3
Discharge	None	Minimal	Moderate	Heavy
Conjunctival redness	None	Minimal	Moderate	Heavy
Size of corneal opacity	0	0% to ≤25%	25% to 75%	75% to 100%
Density of corneal opacity	Iris/pupil margin fully visible	Hazy	Cloudy	Iris/pupil margin not visible
Corneal surface	Smooth, epithelium present	Partial epithelial disruption	Epithelium absent	Descemetocoele

canthus. The eyewash was then vortexed, serially diluted, and plated in triplicate as above.

Grading Scale for Disease Severity

Infected eyes were photographed using a Sony digital camera (DSC-W90; Sony Corp., New York, NY, USA) and graded for

severity of disease pathology using the grading scheme reported by Lee et al.²⁸ with additional modifications for use in the rabbit (Table 1). Eyes were graded on a scale of 0 (none) to 3 (severe) in each of the following five categories: discharge, conjunctival changes, corneal opacity size, corneal opacity density, and corneal surface regularity. Scores in each category

FIGURE 1. Rabbit corneas following 3 to 5 days of inoculated lens wear. (A) Normal cornea at baseline. (B–C) PMMA lenses induced a range of mild to moderate pathology. (B) Early stage infectious keratitis with loss of the full-thickness epithelium and central opacification (arrow). (C) Moderate infectious keratitis with significant conjunctival inflammation, mucopurulent discharge, and peripheral ulceration. (D) In contrast, TA lens wear always induced severe corneal ulceration and stromal melting. (E–H) Three-dimensional reconstructed corneas from IVCM showing increasing severity of pathology and associated cellular infiltration. (E) Normal cornea with an intact epithelium, normal stroma, and endothelium. (F) Edematous cornea with loss of epithelium and mild central inflammatory cell infiltration (corresponding to [B]). (G) Moderate corneal ulceration with increased inflammatory cell infiltration (corresponding to [C]). (H) Severe corneal ulceration with dense inflammatory cell infiltration and stromal melting (corresponding to [D]). Scale bar: 100 μm. Ten-micrometer cryostat-sectioned corneas showing infiltrated neutrophils stained with myeloperoxidase (blue) and nuclei counterstained with propidium iodide (red). Images are representative of above conditions: (I) normal cornea, (J) mild corneal ulceration, (K) moderate corneal ulceration, (L) severe corneal ulceration. Scale bar: 48 μm.

FIGURE 2. Direct visualization of GFP-labeled PA from a severely infected cornea. The presence of GFP-PA confirmed infection by our inoculum. A representative image is shown. Simultaneous DIC images were used to provide a reference for localization within corneal tissue. (A) GFP-PA, $\times 20$. (B) Corresponding DIC image. Scale bar: 76 μm . (C, D) Higher-magnification image of region within the square highlighted in (A). (C) GFP-PA, $\times 63$. (D) Corresponding DIC image. Scale bar: 24 μm .

were summed with a total potential score of 15. A score of 1 to 5 was considered mild, 6 to 10 moderate, and 11 to 15 severe.

Immunofluorescence

Following euthanasia, corneas were excised and fixed in RNase-free 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA). Tissue was embedded in cryo-embedding medium (Fisher Scientific, Waltham, MA, USA) and frozen in liquid nitrogen. Ten-micrometer cryosections were permeabilized in 0.25% Triton X-100 (Sigma-Aldrich Corp.) and washed with PBS. After blocking in 5% normal goat serum (Life Technologies, Grand Island, NY, USA) in PBS, corneas were stained with an antimyeloperoxidase (anti-MPO) mouse monoclonal antibody (Abcam, Cambridge, MA, USA) overnight at 4°C. Corneas were then incubated in an anti-mouse IgG secondary antibody (Alexa Fluor 647; Cell Signaling, Danvers, MA, USA), and nuclei were counterstained with propidium iodide (Cell Signaling). Samples were mounted using an antifade reagent (Prolong Gold; Invitrogen, Grand Island, NY, USA) and imaged using a Leica SP2 laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany). To visualize GFP-labeled *P. aeruginosa*, tissue sections were imaged directly on the laser scanning confocal microscope without further staining. Differential interference contrast (DIC) imaging was used to visualize corneal tissue.

Statistical Analysis

Statistical analysis was performed using Sigma Plot 11.0 (Systat Software, Inc., San Jose, CA, USA). All data are expressed as mean plus or minus standard deviation. For comparison between two groups, an unpaired Student's *t*-test was used. A Mann-Whitney rank sum test was used for non-normal distributions. To determine the difference in the frequency of infection between lens groups, a χ^2 test was used. Statistical significance was set at $P < 0.05$.

TABLE 2. *P. aeruginosa* Harvested From Ocular Surface Washes

PMMA		Tisilfocon A	
Eye Wash CFU	Disease Severity	Eye Wash CFU	Disease Severity
1.09E + 06	Severe	1.71E + 06	Severe
0	Mild	4.00E + 05	Severe
2.24E + 07	Mild	6.67E + 04	Severe
1.95E + 03	Moderate	1.47E + 06	Severe
0	Mild	4.47E + 07	Severe
4.67E + 05	Severe	No sample	Severe

E, exponent.

RESULTS

In the first set of experiments, 12 rabbits were fit on the right eye with rigid lenses composed of either PMMA ($n = 6$) or TA ($n = 6$) inoculated with *P. aeruginosa* and worn for 3 to 5 days. Within this time period, four of the six rabbits fit with PMMA lenses inoculated with *P. aeruginosa* developed clinical signs of infection. This ranged from mild to severe based upon their clinical presentation (Figs. 1B, 1C), which included conjunctival redness and swelling, degree of mucopurulent discharge, and depth and extent of corneal opacity. The two rabbits that failed to show obvious signs of infection (the absence of mucopurulent discharge and corneal opacification) both developed large, central epithelial defects. In the infected eyes, increased inflammatory cell infiltration was evident on the confocal examination (Figs. 1F, 1G). In contrast to PMMA lenses, wear of the TA lens inoculated with *P. aeruginosa* resulted in severe corneal infection in all six rabbits, with extensive corneal opacification and, in some cases, descemetocoele and corneal perforation (Fig. 1D). The severity of corneal opacification resulted in substantial light attenuation during confocal scanning, which precluded full-thickness corneal imaging (Fig. 1H). Neutrophil infiltration in infected tissue was confirmed by MPO staining (Figs. 1J, 1K) and paralleled the IVCN findings.

There was a statistically significant difference in the frequency of infection between the two lens types (66% for PMMA compared to 100% for TA; $P < 0.001$, χ^2 test). For severely infected corneas, direct visualization of GFP-labeled PA showed large numbers of *P. aeruginosa* throughout the full-thickness cornea, with dense fluorescence present on the anterior surface (Fig. 2). In all eyes with moderate and severe disease, PA was recovered from the eye washes performed following lens removal (Table 2).

There was no difference in the time course of disease between the two lens types ($P = 0.447$; Fig. 3A). The degree of disease severity between the two lens types was significant ($P = 0.019$; Fig. 3B), with the TA lens material showing a 1.5-fold increase in severity compared to PMMA. Comparison of bacterial adhesion to each lens polymer showed 26% fewer viable bacteria adherent to PMMA lenses before wear compared to TA; however, this decrease was not significant ($P = 0.190$; Fig. 3C). Similarly, there was a 41% reduction in bacterial adherence to PMMA lenses after wear compared to TA lenses. This difference was not significant ($P = 0.329$; Fig. 3D).

In the second set of experiments, three rabbits in each lens group (PMMA and TA) were fitted with an inoculated lens on one eye and a sterile, control lens on the contralateral eye. Similar to experiment one, rabbits wearing inoculated PMMA lenses showed a range from mild to moderate corneal

FIGURE 3. Corneal pathology and bacterial adhesion. (A) There was no difference in the time to disease onset between lens materials ($P = 0.447$; $n = 6$ TA, $n = 4$ PMMA). (B) For infected eyes, disease severity was significantly worse following wear of the TA lens ($P = 0.019$; $n = 6$ TA, $n = 4$ PMMA). (C) There was a 26% reduction in adhered PA to PMMA lenses compared to TA lenses; however, this was not significant ($P = 0.190$; $n = 10$ per lens group). (D) There was a 41% reduction in PA adhered to PMMA lenses compared to TA lenses after wear, although this difference was not significant ($P = 0.329$; $n = 6$ TA, $n = 4$ PMMA).

pathology. This included a full-thickness corneal epithelial defect with stromal edema (Fig. 4A) and the presence of a corneal opacity with copious mucopurulent discharge (Figs. 4B, 4C). In contrast, wear of inoculated TA lenses resulted in moderate (Fig. 5A) to severe corneal pathology (Figs. 5B, 5C). Representative IVCN images are shown corresponding to the clinical pathology in each figure (Figs. 4D–4F, 5D–5F). Similar to experiment 1, the frequency of infection with PMMA lenses was 66% compared to 100% with TA lenses ($P < 0.001$, χ^2 test). In addition, there were no differences in the time course to disease onset ($P = 0.643$; Fig. 6A) or bacterial adherence to lenses after wear ($P = 0.815$; Fig. 6C). Clinical grading of disease severity also resulted in identical outcomes to experiment 1, with TA lenses showing a 1.5-fold increase compared to PMMA lenses. Due to the low sample size in this latter group (only three rabbits per group), this was not significant ($P = 0.086$, power of 0.342; Fig. 6B). A subsequent power analysis indicated that a minimum of four rabbits per group would have been required to achieve significance as seen in the first cohort.

As expected, wear of the TA lens material did not induce surface epithelial damage, demonstrated by the presence of large, flattened squamous cells (Figs. 7A, 7B). Confocal microscopy through focusing (CMTF) failed to detect any differences in corneal epithelial or stromal thickness. In contrast, PMMA lens wear resulted in varying degrees of damage to the surface epithelium. This ranged from partial cell loss, evident by smaller epithelial cells visualized by IVCN (Fig. 7C) and a full-thickness epithelial erosion (Fig. 7D). Using CMTF, there was an increase in corneal swelling and light scatter compared to before-lens values.

DISCUSSION

In this study we established, to our knowledge, the first reproducible model of infectious keratitis associated with rigid high oxygen transmissible lens wear in the rabbit eye. In the rabbit model, severe infection associated with high oxygen transmissible lens polymers was associated with dense neutrophil infiltration, corneal melting, and perforation. Only two prior studies have reported on the use of contact lenses inoculated with *P. aeruginosa* as a vector to induce infection, both using rodent models.^{17,18} In the first study, rats were inoculated with low- and high oxygen transmissible soft lenses preloaded with a cytotoxic strain of *P. aeruginosa* adherent to the lens and in planktonic form applied topically following lens insertion.¹⁸ The authors reported infection in 30% of the eyes wearing low oxygen transmissible lenses with no evidence of infection developing in the high-oxygen lens group. In a later study, rats were fit with low oxygen transmissible lenses inoculated with *P. aeruginosa*, strain PAO1, under variable conditions.¹⁷ Using this lens material, the authors reported infection in 100% of the rat eyes tested. In the present study, rabbits were fitted with rigid lenses composed of either a non-oxygen transmissible or an ultrahigh oxygen transmissible material. Consistent with earlier reports in the rodent eye, we found that wear of non-oxygen transmissible lenses inoculated with an invasive corneal isolate of *P. aeruginosa* resulted in keratitis in the majority of rabbits tested. Surprisingly, however, the frequency and severity of infection was greatest following wear of the ultrahigh oxygen transmissible lenses. This finding appears to be opposite that of Zhang et al.¹⁸ who were unable to infect the rat cornea during wear of a high oxygen transmissible silicone hydrogel lens. However, there are several considerations with the work by Zhang et al.¹⁸ that prohibits

FIGURE 4. Rabbit corneas following 1 to 3 days of PMMA lens wear. Consistent with the first cohort, PMMA lenses induced a range of mild to moderate pathology. Shown in order of increasing severity, this included a full-thickness epithelial defect with stromal edema (**A**) and moderate corneal ulceration with conjunctival inflammation, copious mucopurulent discharge, and visible corneal opacification (**B**, **C**). (**D–F**) Three-dimensional reconstructed corneas from IVCM corresponding to (**A–C**). Scale bar: 100 μ m.

extrapolation of their results to the present study. This includes the short duration of bacterial challenge, differences in the fit of a soft lens over the rodent cornea, and failure to remove the nictitating membrane prior to lens wear. The presence of a nictitating membrane in the rodent eye can influence both lens centration and movement, key parameters in contact lens fitting.¹⁸

Multiple factors may account for the high infection rates seen in our study. First, due to the large diameter of the rigid lens that is necessary to facilitate lens retention and the reduced blink rate in the rabbit eye, there is little to no tear

exchange under the lens. This is similar to wear of the soft lens in the human eye, with diameters of 14.0 mm that overlap the limbal border. The absence of adequate tear exchange prevents the clearance of debris and cellular by-products from underneath the lens. Previous studies in our laboratory have shown that when challenged with bacteria adherent to the posterior lens surface, there is a robust, subclinical inflammatory response with large numbers of tear film-derived neutrophils accumulated under the lens surface (Robertson DM, unpublished data, 2012). We have further shown that the accumulation of dying neutrophils under the lens rapidly

FIGURE 5. Rabbit corneas following 1 to 3 days of TA lens wear. As seen in cohort 1, wear of inoculated TA lenses resulted in moderate (**A**) to severe pathology (**B**, **C**). (**D–F**) Three-dimensional reconstructed corneas from IVCM corresponding to (**A–C**). Scale bar: 100 μ m.

FIGURE 6. Corneal pathology and bacterial adhesion. (A) There was no difference in the time to disease onset between lens materials ($P = 0.643$; $n = 3$ per lens group). (B) Disease severity scores paralleled those in experiment 1, with TA lens wear exhibiting greater pathology; however, due to the low sample size, this difference was not significant ($P = 0.086$; $n = 3$ per lens group). (C) There was no difference in PA adherence to the lens surface following lens wear in either group ($P = 0.815$; $n = 3$ per lens group).

FIGURE 7. In vivo confocal microscopy of corneal surface following lens wear. In contrast to TA lens wear, lens-induced hypoxia was associated with corneal epithelial surface damage. (A, B) Three-dimensional reconstruction of the rabbit cornea after TA lens wear. The surface epithelium showed large corneal epithelial surface cells, indicating the absence of mechanical surface damage. Analysis of confocal microscopy through focusing (CMTF) reflected light-intensity peaks did not show any signs of corneal swelling. (C, D) Three-dimensional reconstruction of the rabbit cornea following PMMA lens wear. Varying degrees of epithelial damage were evident on IVCN exam. (C) The size of the surface epithelial cells was visibly reduced, indicating loss of the surface epithelium (arrow). This was accompanied by mild stromal swelling with increased corneal light scatter in the anterior cornea (*). The CMTF light-intensity peaks confirmed a 15% increase in stromal thickness compared to before-lens values due to lens-induced hypoxia. (D) Full-thickness corneal erosion (arrow) with increased corneal swelling (40%) and light scatter (*) compared to before-lens values. Scale bar: 100 μm .

accelerates bacterial colonization of the lens surface and bacterial uptake into the corneal epithelium.²² This occurs as a result of electrostatic interactions between the remnants of extracellular debris released by necrotic inflammatory cells.^{29,30} In our model, the absence of adequate tear exchange under the rigid lens thus creates a reservoir for the build-up of inflammatory debris and formation of these neutrophil-derived microbial scaffolds. Consistent with this data, inoculated rigid lens wear for 16 hours in the cat eye showed differences in *P. aeruginosa* adhesion that was dependent on lens geometry, with orthokeratology lenses that create significant tear pooling under the lens in the midperiphery having higher colonization rates than alignment fit lenses that evenly distribute tear fluid under the lens.³¹

A second factor that may influence the severity of infectious keratitis found in this study is the potential for lens material surface properties to modulate bacterial adherence.³² Since the lenses used in this study are rigid lenses, they are reusable following sterilization, and over time, scratches and surface roughness may lead to differences in bacterial adherence and lens colonization. While we found a slight trend toward an increase in bacterial adherence to the ultrahigh oxygen transmissible lens material, this did not reach statistical significance. Thus, increased adherence to the lens surface did not appear to be a driving factor in infection severity. Interestingly, inoculum levels on lenses following removal were similar between the first and second cohort, despite an apparent acceleration in the development of disease in the latter group. It is possible that the vortexing method employed to disperse or dislodge adherent bacteria may have resulted in clumping that could have adversely affected the bacterial counts after wear. However, since these two groups underwent lens wear several months apart and there were no differences in the amount of viable bacteria colonized on lenses after wear, other environmental factors or biological variables may also play a role. Further studies are needed to investigate the impact of inoculum size on the early stages of infection and clinical outcomes in the rabbit model.

The impact of inoculation parameters has been previously investigated in a rodent model using low oxygen transmissible lenses.¹⁷ In that study, Tam and colleagues¹⁷ used both high and low test inoculums and reported no difference in the rate or severity of infection. As in our study, disruption of the corneal epithelial surface failed to accelerate disease. Instead, biofilm-coated lenses transferred from infected to naïve eyes shortened the duration of time from lens insertion to the establishment of severe keratitis, suggesting that variables associated with bacterial adaptation and not inoculum level,

was a precipitating factor.¹⁷ While the extent of biofilm formation on the different lens polymers was not assessed in this study, as the steep curvature of the rigid lens prevented successful microscopic examination of the lens surface, the finding that infection rate was not dependent on inoculum level or mechanical surface damage is in agreement with ours.

A third consideration in this study is the use of an invasive corneal isolate of *P. aeruginosa*, which has been shown to internalize in corneal epithelial cells via lipid raft-mediated uptake.¹² Increased rates of *P. aeruginosa* internalization have been reported in response to hypoxic lens wear and the use of chemically preserved contact lens solutions.^{11,12,33} This is important in the lens-wearing eye where the ability of infected cells to desquamate is inhibited, resulting in the retention of infected cells at the corneal surface and a corresponding increase in epithelial-associated microbial bioburden.^{34,35} The ability of cytotoxic strains to use cellular-derived scaffolds to facilitate colonization of the lens during wear and the subsequent effect on their ability to penetrate and traverse the intact corneal epithelium has not yet been investigated. As cytotoxic strains of *P. aeruginosa* do not internalize but instead induce cell death and damage at the epithelial surface, the increase in cellular debris derived from corneal epithelial cells may exacerbate the bacterial response under the lens. Further studies are necessary to investigate potential differences in the mechanisms of infection between invasive and cytotoxic strains in the presence of high oxygen transmissible lens wear.

Finally, the rabbit model has been widely used to demonstrate the effects of contact lenses on the corneal epithelium.³⁶ This includes a reduction in basal cell mitotic rates in the central corneal epithelium and a corresponding delay in the vertical migration of epithelial cells from the basal layer to the epithelial surface.^{37–40} While basal cell mitosis has been shown to be mediated as a function of lens-induced hypoxia, the well-documented reduction in the ability of surface epithelial cells to undergo apoptotic desquamation into the precorneal tear fluid is independent of lens oxygen and, more importantly, has been shown to correlate with human clinical findings confirming a reduction in epithelial cell shedding as a consequence of lens wear.^{14–16,34,35,41,42} These same studies have shown that bacterial binding to exfoliated corneal epithelial cells in response to lens-induced hypoxia directly correlates with bacterial binding to the damaged corneal epithelium in the lens-wearing rabbit eye, validating this as a clinical metric for assessing lens-induced corneal surface damage.^{43–45} The ability to directly translate bench data obtained from the rabbit eye to the human corneal surface supports the use of the rabbit contact lens model, as used in this study. It is important, however, when interpreting these results to keep in mind that the rigid lenses used in the present study are larger in size and do not move as they would in the human eye. Thus, the normal tear-flushing effects that occur under the rigid lens in the human eye are likely a key factor in maintaining lens sterility and protection from bacterial adherence to the epithelium.⁴⁶

Based upon several longitudinal studies evaluating bacterial binding to corneal epithelial cells following lens wear in the human, it was predicted that use of ultrahigh oxygen transmissible silicone hydrogel lens materials would eliminate hypoxic damage to the corneal epithelium and confer increased ocular protection.^{14–16} This includes a reduction in the risk associated with 30-night compared to 6-night extended wear. While the latter prediction has been confirmed, it is now well established that the clinical use of silicone hydrogel lenses has not reduced the annualized incidence of infection. As soft lenses exhibit minimal tear exchange compared to their rigid lens counterparts, our data would argue that *P. aeruginosa*

trapped under the lens surface, either soft or rigid, in the absence of adequate tear exchange overrides the protective effects of increased lens oxygen transmission. Furthermore, as shown by our lab and others, the confounding effects of chemically preserved solutions that are frequently encountered in soft contact lens wear may also contribute to increased bacterial adhesion and invasion into corneal epithelial cells.^{33,47–50}

Importantly, our control studies are consistent with prior reports using high oxygen transmissible lens materials in the rabbit, which demonstrate the absence of any lens-induced corneal epithelial surface damage, whereas low or non-oxygen transmissible lens wear resulted in loss of one or more layers of superficial squamous cells.⁴³ The loss of surface epithelium arising from lens-induced hypoxia leads to exposure of deeper, less-differentiated cells. The biological response of this newly exposed cell population, compared to more mature surface cells that may already be committed to apoptotic-mediated desquamation, is potentially very different. Similarly, the presence of serum-derived factors in tear fluid and neural stimulation in a damaged hypoxic corneal epithelium compared to the healthy epithelial surface under the high-oxygen lens may result in the activation of wound-healing pathways and the upregulation of additional defensive factors. Further investigations into the behavior of these individualized cell layers and neural protective factors under the lens in the presence and absence of bacteria are required.

In summary, we have established, to our knowledge, the first reproducible model of contact lens-related infectious keratitis using contemporary ultrahigh oxygen transmissible rigid lens materials. These findings suggest that adequate tear clearance under the lens may represent an important inhibitory mechanism to enhance bacterial clearance and prevent biofilm formation in the early stages of infection. These findings further point toward the existence of different pathophysiological mechanisms whereby changes under the lens in the absence of frank hypoxic damage result in *P. aeruginosa* infection in the otherwise healthy corneal epithelium. The ability of bacteria-colonized lenses to induce infection in the absence of preexisting corneal surface damage in both the rabbit and rat increases the likelihood that these animal models reflect the underlying processes that occur in humans. This also highlights the importance of using contact lens-wearing in vivo animal models for investigations into the key biological factors that mediate bacterial adaptation and host response during lens wear.

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