Use of Polymyxin as an Endotoxin Blocker in the Prevention of Diffuse Lamellar Keratitis in an Animal Model

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ABSTRACT

PURPOSE: To determine whether bacterial endotoxin, lipopolysaccharide (LPS), could induce diffuse lamellar keratitis (DLK) in an animal model and whether DLK could be prevented by endotoxin blockers such as polymyxin.

METHODS: Laser in situ keratomileusis (LASIK) flaps were created in rabbit eyes. The stromal bed was treated with 20 μg of *Burkholderia cepacia* LPS or balanced salt solution (BSS). Development of DLK, histological degree of inflammation, and presence of LPS detected by anti-LPS antibody were evaluated after 48 hours. In a second experiment, all eyes received LPS and were randomly assigned to receive either polymyxin in the form of two drops of Polytrim (Allergan, Irvine, Calif) on the stromal bed or two drops of BSS.

RESULTS: In the animal model study, LPS was significantly associated with the development of DLK ($P<.05$, n=30). Infiltration with polymorphonuclear cells and presence of DLK were found in LPS treated eyes but not in controls. In the second experiment, 4 (27%) of 15 eyes that received polymyxin in addition to LPS developed DLK compared to 18 (95%) of 19 eyes that received only LPS ($P<.05$, n=34). There was a trend towards higher flap displacement in polymyxin treated eyes but this was not significant ($P=.07$).

CONCLUSIONS: Diffuse lamellar keratitis in a rabbit model can be caused by bacterial endotoxin (LPS). Endotoxin blockers, such as polymyxin, are effective in decreasing the incidence of endotoxin-induced DLK in a rabbit model. [*J Refract Surg*. 2005;21:152-157.]

Diffuse lamellar keratitis (DLK) is regarded as a nonspecific, inflammatory condition occurring after laser in situ keratomileusis (LASIK).[1] Etiology usually is considered to be multifactorial.[2] We previously suggested bacterial endotoxin as a cause of DLK[3] and postulated that the source of endotoxins might be gram-negative biofilms in sterilizer reservoirs.[4] We have also shown from clinical, epidemiological, and microbiological data that lipopolysaccharide (LPS), more specifically, may be an important cause of cluster or outbreak DLK.[4]

Endotoxin or LPS is recognized as a potent initiator of inflammation and is responsible for septic shock syndrome.[5] Schultz et al[6] previously demonstrated infiltration with polymorphoneutrophils after LPS deposition on abraded corneas in an animal model.

We developed an animal model to determine whether LPS derived from *Burkholderia cepacia*, found in sterilizer biofilms during an outbreak of DLK, could cause DLK in a rabbit model. We chose to use LPS from *B cepacia* as we have found this bacteria and *Ralstonia pickettii* to be the two most frequently isolated gram-negative bacteria in sterilizer reservoirs in 10 investigated DLK outbreaks.[7] The use of this LPS

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should more closely simulate the clinical experience than commercially available LPS.

Lipopolysaccharide is a constituent of the outer membrane of gram-negative bacteria and is ubiquitous in moist environments where gram-negative bacteria are present.[8] Thus developing endotoxin blockers to be used intraoperatively during LASIK may provide the ability to prevent the development of LPS-induced DLK. Peptide antibiotics such as polymyxin are effective blockers of LPS-induced inflammation.[5] Therefore, we also planned to determine whether polymyxin applied to the stromal bed during LASIK could prevent or limit the development of DLK in a rabbit model.

MATERIALS AND METHODS

ANIMAL MODEL STUDY

New Zealand white rabbits were used in the first study (N=18). The animals were housed and cared for in accordance with the Canadian Council on Animal Care Guidelines for the Care and Use of Experimental Animals.[9] The study protocol was reviewed and approved by the Institutional Review Board (Institutional Animal Care Committee of the University of Calgary). Purified bacterial lipopolysaccharide (LPS) was prepared from *Burkholderia cepacia* isolated from the inner tubings of a STATIM (SciCan, Mississauga, Ontario, Canada) sterilizer reservoir during an outbreak of DLK previously investigated by the authors.[2] The *B cepacia* was identified by the *B cepacia* Laboratory, British Columbia Children's Hospital, which is the Canadian reference center for *Burkholderia*. The quantification of the LPS was performed at the Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada.

Each rabbit was anesthetized with 4% halothane and oxygen (2 L/min). Body temperature was maintained with a circulating water unit. The preoperative drops used were chosen based on those used by TLC, The Laser Centre, Vancouver, British Columbia, and were as follows: dexamethasone/tobramycin (Tobradex; Alcon Laboratories, Ft Worth, Tex), ofloxacin 0.3% (Ocuflox; Allergan, Irvine, Calif), diclofenac sodium 0.1% (Voltarin; CIBA Vision, Mississauga, Ontario, Canada), and proparacaine hydrochloride 0.5% (Alcaine, Alcon); each was applied twice 15 minutes preoperatively. The microkeratome used was the Automatic Corneal Shaper (Bausch & Lomb, Irvine, Calif) using a 160-µm plate. A new blade was used for each animal with the keratome head irrigated with Alcaine between eyes.

After the LASIK flap was cut, it was inspected and if satisfactory, lifted and 20 µL of balanced salt solution (BSS) (Alcon) was placed on the stromal bed and the flap replaced. Tobradex, Ocuflox, and Voltarin drops were applied and lids taped while the second eye was operated on. The second eye treated on each animal received 20 µg purified *B cepacia* LPS in 20 µL of BSS. We used this quantity of LPS based on a pilot dose response study (unreported) that we performed using the identical LPS. In this study, flap necrosis was found above 50 µg and a variable DLK response was found at 10 µg. This part of the study was not randomized as the first eye was used as a control to minimize the possibility of contaminating the fellow eye with LPS. However, the observer was not aware of which eye had been treated first and thus was masked. The postoperative drop regimen was identical to that used in the first eye.

The animals were monitored continuously by a veterinarian for the 48-hour duration of the experiment and received analgesia using butorphanol 2 mg/kg body q6h. Tobradex drops were applied 6 hours after surgery and twice the following day. At 48 hours postoperatively, each animal was anesthetized as above. Each eye was examined by a corneal surgeon experienced in managing DLK and scored on a 4-point scale: DLK grade 0 = no inflammation, grade 1 = diffuse infiltrate not involving the central cornea, grade 2 = diffuse infiltrate involving the entire flap interface, grade 3 = grade 2 but with a dense infiltrate, and grade 4 = severe infiltrate with marked edema, stromal necrosis, or both. Flap displacement was also noted. Each eye was photographed during the slit-lamp examination using a Nikon FS-3 Photo slit-lamp (Nikon, Toronto, Ontario, Canada)

Following slit-lamp photography, euthanasia was performed using intravenous overdose of sodium pentobarbital and each eye was removed and fixed in 10% neutral buffered formalin. The tissues were trimmed, dehydrated, and embedded in methacrylate (JB4 Immunobed). Sections (3 µm) were cut and stained with methylene blue and basic fuchsin. Further specimens were used for immunochemical labeling of *B cepacia* LPS in the tissue sections using high titre, anti-*B cepacia* LPS serum previously raised in mice. Analysis was performed using chi square ($P<.05$) on the presence or absence of DLK.

Sterilization of the instruments was performed using a STATIM sterilizer with the external reservoir modification in place to minimize the possibility of contamination arising from the sterilizer reservoir (Holland SP, Mathias R, Morck DW, Slade SG. Control of outbreaks of diffuse lamellar keratitis with sterilizer reservoir modification. *Ophthalmology*. In review). In cases with intraoperative complications, the complication was managed, as would be the normal practice for a human eye, and the eye was excluded from the study although the fellow eye was treated.

TABLE 1

Rabbit Model of Endotoxin-induced Diffuse Lamellar Keratitis (DLK)

	Left Eye (LPS)	Right Eye (Sterile BSS)
DLK (>grade1)	16*	0*
No DLK (grade 0)	0*	14*
Total	16	14†
Displacements	2	2

LPS = lipopolysaccharide, BSS = balanced salt solution
**Indicates significant difference (P<.05).*
†Surgical loss of two eyes (1 no flap, 1 free flap).

Polymyxin Study

In the polymyxin study, New Zealand white rabbits were also used in accordance to guidelines.[9] Each eye was randomly assigned to receive LPS or LPS and polymyxin B and the experiment was double masked. Polytrim was used as it is a commercially available preparation of polymyxin B and trimethoprin. In each eye, 20 µg of purified *B cepacia* LPS in 20 µL of BSS was placed on the stromal bed after creation of the LASIK flap. After 20 seconds, two drops of either BSS or Polytrim were placed on the stromal bed and the flap replaced. The postoperative care given to the rabbits was identical to that given in the first study with the same measurements taken.

In vitro assessment of *B cepacia* LPS was done by a measurement of tumor necrosis factor-alpha (TNF-α) production by RAW 264.7 macrophages. The murine macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Manassas, Va). The cell line was maintained in Dulbecco's modified Eagle medium (Life Technologies, Burlington, Ontario) supplement with 10% fetal calf serum. RAW 264.7 cells were seeded into 24 well plates at a density of 106 cells per well in Dulbecco's modified Eagle medium and incubated at 37°C in 5% CO_2 overnight. Dulbecco's modified Eagle medium was aspirated from cells grown overnight and replaced with fresh medium. Either *B cepacia* LPS or *B cepacia* LPS and polymyxin B were incubated with the cells for 6 hours at 37°C in 5% CO_2. The supernatants were removed and tested for TNF-α production by enzyme-linked immunosorbent assay as per manufacturer's directions (R&D Systems Inc, Minneapolis, Minn). The experiments were repeated three times and the results were analyzed as the average percent inhibition of TNF-α ± standard error.

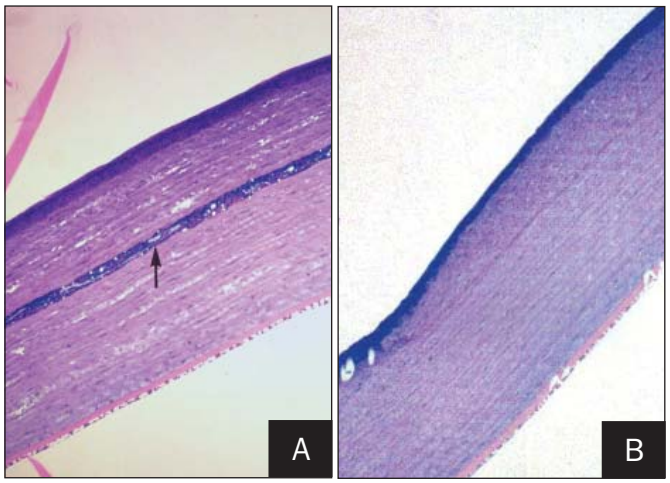

Figure 1. A) Light photomicrograph of a methacrylate embedded Lee's methylene blue basic fuchsin stained thin section of cornea demonstrating the diffuse infiltrate of polymorphoneutrophils (arrow) in the stromal bed in a rabbit treated with LPS (original magnification ×100). **B)** Light photomicrograph of a methacrylate embedded Lee's methylene blue basic fuchsin stained thin section of corneal stromal bed in a rabbit treated with BSS. Distinguishing the actual corneal flap is difficult (original magnification ×100).

RESULTS

Animal Model Study

A total of 30 eyes from 16 animals were available for analysis. Intraoperative complications included one free flap that was sutured in position and an epithelial defect, which occurred without flap creation. Two animals had bilateral flap displacements. Rabbit eyes treated with LPS from *B cepacia* under the LASIK flap were significantly more likely ($P<.05$) to develop DLK than those treated with BSS alone (Table 1). Histological assessment showed diffuse infiltrate with polymorphoneutrophils of the stromal bed and flap in eyes that had been treated with LPS and only a minimal reaction in those treated with BSS alone (Fig 1). Immunohistochemical labeling for *B cepacia* LPS showed prominent staining for LPS in the eyes that had been exposed but not in the BSS control eyes (Fig 2). Slit-lamp examination showed presence of DLK in an LPS-treated eye versus absence of DLK in a control eye.

Polymyxin Study

Incidence of endotoxin-induced DLK was significantly less in eyes that received polymyxin B (as Polytrim) and LPS compared to controls that received BSS and LPS ($P<.05$) (Table 2). There was a trend towards higher flap displacement with polymyxin, but this was not significant ($P=.07$). Flap displacement was defined when causing the exposure of >1 mm of the stromal bed. There were insufficient eyes for analysis by grade

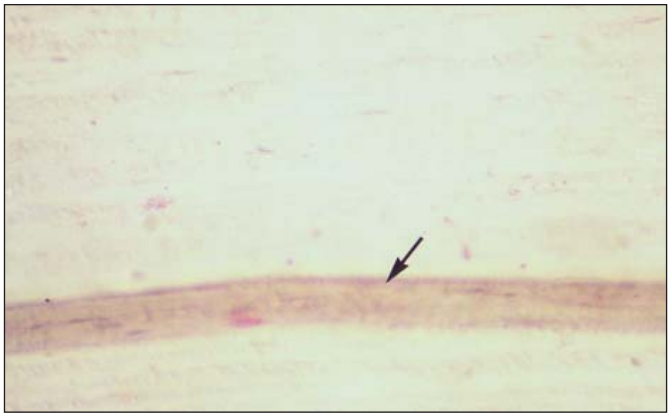

Figure 2. Light photomicrograph of a methacrylate embedded immunohistochemically stained (anti-LPS antibody and detection conjugate) thin section of cornea demonstrates the labeling of LPS (arrow) in the stromal bed following flap creation. Note the obvious identity of the flap due to the LPS labeling (original magnification ×200).

TABLE 2

Comparison of the Incidence of Diffuse Lamellar Keratitis (DLK) in Endotoxin Treated Eyes and in Polymyxin B Exposed Endotoxin Treated Eyes in Experimental Rabbits

	LPS and Polymyxin	LPS
DLK (>grade 1)	4*	18*
No DLK (grade 0)	11*	1*
Total	15	19
Flap displacements	5	1

LPS = lipopolysaccharide
**Indicates significant difference (P<.05).*

of DLK. No intraoperative complications occurred, although only 34 eyes were available for analysis, as six displaced flaps were excluded. Histological sections of corneas exposed to endotoxin (see Fig 1A) had more inflammation than those exposed to both endotoxin and polymyxin (Fig 3). Silt-lamp examination showed an absence of DLK in a cornea exposed to LPS and polymyxin as compared to the presence of DLK in a cornea treated with LPS alone.

In Vitro Study

In vitro results showed that TNF-α was induced at LPS concentrations of 0.1 to 10 µg/mL. When polymyxin B (1 µg/mL) was also added, a qualitative decrease occurred (67%±1.5% SE) in the amount of TNF-α induced by *B cepacia* LPS.

DISCUSSION

Bacterial endotoxin or lipopolysaccharide (LPS) has been suggested as one of the causes of DLK.[2,3] This preliminary study has shown that LPS from *B cepacia* can cause diffuse lamellar inflammation under the LASIK flap in a rabbit model. These results support the theory that bacterial endotoxin or LPS is a cause of DLK. Similar findings have been reported by Peters et al.[11] A potential weakness in our LASIK rabbit model is that no laser was performed on the exposed stromal bed. However, despite this, DLK developed in response to LPS without laser treatment or further mechanically induced keratocyte trauma. The study design could also be improved if it was fully randomized rather than the consistent use of the first eye as the control, although the observer was masked as to which eye was treated first. The experiment was designed in this way to minimize the risk of LPS contamination of the second eye. It should also be noted that no animal was followed beyond 48 hours and therefore we were unable to determine whether any risk of late onset DLK exists.

Lipopolysaccharide occurs in the outer membrane of gram-negative bacteria and is recognized by the host immune system, which initiates cellular activation resulting in the release of cytokines and other inflammatory mediators.[8] The features of DLK are consistent with an endotoxin-induced inflammation involving rapid recruitment of polymorphoneutrophils to the exposure site. Schultz et al[6] developed an animal model of endotoxin-induced inflammation using LPS from *Escherichia coli* deposited in the corneal stroma through epithelial abrasion. They were also able to block infiltration of polymorphoneutrophils into the cornea by using an anti-CD18 polyclonal antibody. Lipopolysaccharide was still found along the abrasion but the cellular uptake was abolished. Thus, we believe that these results, in addition to the animal model described by Peters et al[10] and herein, provide further evidence of the importance of bacterial endotoxin in the development of DLK in humans.

We previously reported outbreak or cluster DLK associated with gram-negative biofilms in sterilizer reservoirs.[2] We were able to control the initial outbreak by changing cleaning protocols of the sterilizer reservoirs to minimize the chance for endotoxin contamination of the distillate entering the autoclave. During the reported outbreak, the only variable statistically associated with a reduction in DLK incidence was the use of the sterilizer and whether it had been subject to biofilm control measures. Results of this study may give

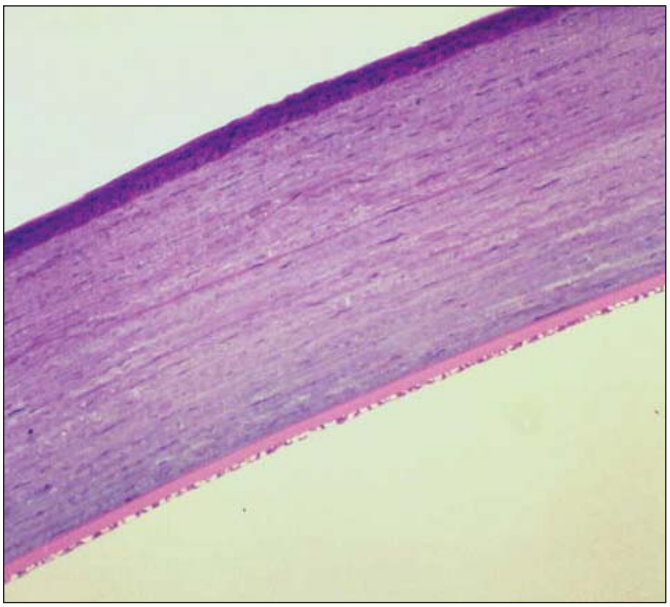

Figure 3. Light photomicrograph of a methacrylate embedded Lee's methylene blue basic fuchsin stained thin section of corneal stromal bed in a rabbit treated with LPS and polymyxin following flap creation. Distinguishing the actual corneal flap is difficult (original magnification ×100).

indirect evidence of endotoxin as a cause of outbreak DLK. We do not know at this stage what role endotoxin might play in sporadic cases of DLK and what other cofactors may be operational in causing outbreak DLK. However, it would appear prudent to attempt to limit endotoxin exposure of the stromal bed during LASIK whenever possible.

Our second study suggests that it is possible to prevent the development of LPS-induced DLK by the use of polymyxin B (eg, Polytrim) on the stromal bed at surgery. We believe the rabbit endotoxin-induced DLK model is a valid method to test this hypothesis. Thus, it may be possible in clinical LASIK to use polymyxin on the stromal bed to lessen the risk of developing DLK. We used Polytrim as the vehicle for polymyxin as this is a commercially available drop and trimethoprin is not known to have LPS blocking capability. We had initially considered using polymyxin as drops postoperatively but decided to use it only on the stromal bed during LASIK to minimize the potential epithelial toxicity that may occur with postoperative application.

There has been considerable interest in developing endotoxin blockers to reduce the incidence and severity of septic shock syndrome.[4] Attention has been concentrated on developing molecules that will bind LPS and prevent the development of the cascade of inflammation resulting in induction of TNF and cell death.[8] The ability of agents such as polymyxin B to bind LPS is not well understood and systemic use of polymyxin B is limited due to its toxicity, especially renal. Alternative antimicrobial peptides that bind or limit the action of LPS include gramicidin S and colymycin, which appear to affect the cytoplasmic membrane by different mechanisms.[11] It is likely that the development of further synthetic peptides with similar efficacy to polymyxin B and less toxicity may become clinically available[5] and offer potential for use in LASIK.

Other potential endotoxin blockers at the stromal bed include polyclonal antibodies such as anti-CD18 used to block infiltration of polymorphoneutrophils into the stroma following corneal abrasion treated with LPS.[6] A difficulty with this approach is that it may be necessary to raise species-specific antibodies and this would limit the practicality of using antibodies applied to the stromal bed.

In the present study, the most common postoperative complication was flap displacement, and there was a trend towards higher flap displacement in Polytrim-treated eyes versus controls. Flap displacement appears to be a problem with rabbit LASIK. This is probably due to the animals rubbing their eyes, as we encountered this in both the animal model and polymyxin portions of this study. We doubt whether this would be a factor in the use of Polytrim in human LASIK.

Diffuse lamellar keratitis is a rare complication of LASIK surgery and carries a good prognosis if closely managed (Holland SP, Mathias R, Morck DW, Keith E, Chiu J, Slade SG. Diffuse lamellar keratitis: clinical features and outcomes. *Ophthalmology*. In review). Thus any routine method adopted for the prevention of DLK must meet a high standard of therapeutic safety. Until further clinical information is available on the use of polymyxin intraoperatively, we would not recommend its routine use in human LASIK. Although in a DLK outbreak, using an endotoxin blocker such as polymyxin on the stromal bed as part of the drop regimen may be a consideration. Peters et al[12] reported similar success in preventing DLK with the use of polymyxin B in a rabbit model.

Effective prevention of DLK, particularly that occurring in outbreaks, requires a further understanding of the etiology. Our group and others have provided some evidence for the importance of bacterial endotoxin (LPS) as an etiological factor.[2] Our group has investigated the cause and prevention of DLK occurring in 51 outbreaks in 38 clinics including recurrences.[13] We achieved outbreak control in many of these by limiting access of endotoxin, from gram-negative bacteria in sterilizer biofilms, to the stromal bed through instruments. Secondary levels of DLK prevention include using endotoxin blockers applied to the stromal bed

at surgery. Our animal study confirms the efficacy of polymyxin applied as Polytrim in a rabbit model. An alternative approach would be to apply steroid drops on the stromal bed after the laser ablation in LASIK, as this has also been shown to effectively reduce the incidence of DLK.[14] Potential difficulties with this approach include reducing immune response at the stromal bed and the theoretical risk of increasing the possibility of microbial infection.

We have shown that DLK can be induced by LPS in an animal model. Furthermore, this animal model has allowed us to show that polymyxin applied to the stromal bed after creation of the LASIK flap is effective at reducing the development of DLK and may have an application in limiting the development and/or severity of DLK in a clinical setting.

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