In Vitro Model of Infectious Crystalline Keratopathy: Tissue Architecture Determines Pattern of Microbial Spread

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PURPOSE. To develop an in vitro model of infectious crystalline keratopathy using human corneal buttons and to test the hypothesis that the compactness of the corneal stroma determines the pattern of microbial spread.

METHODS. Twenty human corneal buttons obtained after penetrating keratoplasty for keratoconus (KC) and eight human corneal buttons obtained from eye bank (EB) donor eyes were maintained in organ culture. Fourteen buttons (10 KC and 4 EB donors) were maintained in a turgid state (swollen, edematous) and 14 in a nonturgid state (compact, normal state of deturgescence) by the omission or addition of 5% dextran to the culture medium. Eight KC and four EB nonturgid buttons and eight KC and four EB turgid buttons were inoculated with Streptococcus viridans (Lancefield group G, gram-positive) organisms. Two KC nonturgid and two KC turgid buttons were inoculated with Klebsiella oxytoca (gram-negative) organisms. Bacterial migration and spread in the tissue were observed by light and electron microscopy.

RESULTS. Of the nonturgid buttons, six KC buttons and all four EB buttons inoculated with S. viridans and both KC buttons inoculated with K. oxytoca demonstrated an arborizing, crystallike pattern of bacterial spread. In the turgid buttons, five KC and all four EB buttons inoculated with S. viridans and both KC buttons inoculated with K. oxytoca demonstrated globular, amorphous colonies. This was in complete contrast to the needlelike branching appearance seen in nonturgid corneal buttons. Electron microscopy confirmed an interlamellar spread of the bacterial colonies.

CONCLUSIONS. This is the first in vitro model of bacterial keratitis. It demonstrates that the pattern of spread of bacteria within corneal tissue is largely determined by the compactness of the corneal stroma. Altering tissue architecture changed the pattern of bacterial migration and spread. This model has considerable potential in further understanding host-microbe interactions and microbial spread that occurs during infection.

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Corneal Buttons

The protocol adhered to the tenets of the Declaration of Helsinki, and informed consent was obtained from all participants. Twenty corneal buttons (7.5–8 mm in diameter), obtained at the time of penetrating keratoplasty from patients with keratoconus (KC) were used in the study. The patients had a clinical and topographic diagnosis of KC. There were 14 men and 6 women aged between 23 and 42 years. Sixteen patients were wearing contact lenses, but none had corneal infections or ulcers at any time. The reasons for corneal transplant were intolerance to contact lens (n = 5), inability to achieve adequate contact lens fit (n = 11), and unwillingness to consider contact lens wear (n = 4). Patients were randomly selected, but individuals with excessive scarring or a history of acute hydrops were excluded. Host trephination diameter was 7.5 mm in 14 patients and 8 mm in 6 patients. In addition, 8 corneal buttons (8 mm diameter) were obtained from eye bank (EB) donor eyes. The corneal buttons were left to equilibrate for 48 hours in Eagle’s minimum essential medium (MEM; Gibco-Life Technologies, Paisley, Scotland, UK) with 2% fetal calf serum, 10 mM HEPES buffer, and 5% dextran (10 KC and 4 EB buttons) and in the same medium without dextran (10 KC and 4 EB buttons), at 25°C. Medium with dextran maintained the corneal buttons in a compact, nonturgid state, whereas medium without dextran allowed the corneal stroma to imbibe water and become swollen or turgid.

Corneal-Thickness Measurement

An ultrasound pachymeter (Bausch and Lomb, Rochester, NY) was used to measure thickness of corneal buttons after equilibration in the respective organ culture media for 48 hours. The mean thickness of the
nonturgid buttons was 601 ± 26 μm (range, 546–655). The mean thickness of the turgid buttons was more than 1000 μm. The ultrasound pachymeter used has a maximum range of 1000 μm. All turgid buttons measured 1000 μm (or more); therefore, it was not possible to determine the SD for this group. In the patients with KC, preoperative corneal thickness measurements were made at the apex and midperiphery (site of inoculation). The mean was 461 ± 31.5 μm (range, 410–510) at the apex and 545 ± 33.1 μm (range, 495–592) at the midperiphery.

**Bacterial Viability in Medium**

*Streptococcus viridans* (Lancefield group G, gram-positive) and *Klebsiella oxytoca* (gram-negative) organisms were suspended in phosphate-buffered saline (PBS; 1 × 10⁷ organisms per milliliter). Bacterial viability was checked in the organ culture medium in which the corneal buttons were to be maintained, to ensure that the medium, with and without dextran, supported the growth of both species of organism in the experimental conditions. For this purpose, 100 μl of the suspension of each of the organisms was inoculated into separate culture tubes containing 10 ml of medium each, and maintained at 25°C, 32°C, and 37°C. Bacterial growth of both species was confirmed at all temperatures, with optimal growth occurring at 25°C. This was therefore the temperature at which the experiments with corneal buttons were conducted.

**Inoculation of Corneal Buttons and Organ Culture**

Fifty microliters (1 × 10⁷ organisms per milliliter) of bacterial suspension was injected into the corneal stroma at the midperiphery of the button. A tuberculin syringe with a 25-gauge needle was used. The needle was introduced from the endothelial side, under a laboratory microscope. The inoculation was undertaken in sterile conditions. Eight KC and four EB nonturgid buttons and eight KC and four EB turgid buttons were each inoculated with *S. viridans* (Lancefield group G) organisms. Two nonturgid KC and two turgid KC buttons were each inoculated with *K. oxytoca* organisms. The buttons were placed in sterile tubes containing 10 ml of the respective medium and maintained at 25°C. The medium was changed every 24 hours and the buttons examined daily, by light microscopy, to progressively document growth of the bacterial colonies. Photographs were taken at ×40 magnification.

**Electron Microscopy**

Before fixation, a sample of the stromal bacterial colonies was taken for bacterial culture, to confirm that the identity of the bacteria was the same as the one inoculated. Between 3 and 14 days, the buttons were fixed in 2% glutaraldehyde and processed for light and electron microscopy (transmission and scanning). Sections for light microscopy were stained with toluidine blue. All samples conformed in identity to the inoculated organisms.

**RESULTS**

**Nonturgid Buttons**

A classic arborescent appearance was observed in six of the eight nonturgid KC and all four EB buttons inoculated with branching network seen in compact corneal buttons (wholemount; magnification, ×40). (C) Light micrograph of a section of nonturgid cornea (toluidine blue). The compact regular arrangement of the lamellae with interlamellar spread of the bacteria is clearly visible. (D) Light micrograph of a section of turgid cornea (toluidine blue). The lamellae are widely spaced with globular rounded areas of bacterial colonization. This is in complete contrast to the needlelike branching pattern seen in nonturgid corneas.
S. viridans (Lancefield group G). The other two did not show any growth. Both the nonturgid KC buttons inoculated with K. oxytoca also demonstrated the arborescent pattern. A striking, well-demarcated, branching, crystallike network of bacterial colonies was easily visible with standard light microscopy at low magnification of wholemounts (Fig. 1A) and sections (Fig. 1C). The main trunks had a segmented or beaded pattern with finely tapered, needlelike terminal ends. The secondary branches also demonstrated fine tapering terminal ends, but the segmentation was less apparent. The pattern became visible within days 3 through 7 after inoculation and persisted with further branching and subbranching over the following period (7 days maximum) of observation.

Scanning electron micrograph views of the horizontally split corneal buttons showed the tapering ends of bacterial colonies forming spikes that were aligned in the direction of the underlying stromal lamella. The sharply tapering tip gives the condition its characteristic clinical pattern. (B) Scanning electron micrograph of an inoculated turgid corneal button. Islands of colonies of bacteria can be seen in spaces between the stromal lamellae. The stromal lamellae are widely separated, and the colonies of bacteria show a diffuse growth pattern.

Turgid Buttons

In turgid corneas, light microscopy revealed a completely different growth pattern with globular, amorphous colonies within the stroma (five of eight KC buttons and all four EB buttons inoculated with S. viridans and both KC buttons inoculated with K. oxytoca; Figs. 1B, 1D). The remaining three buttons did not show any growth.

With scanning electron microscopy, the bacterial colonies were visible in large lacunae among the more widely spaced stromal lamellae, without a definite leading point (Fig. 2B). Light microscopy (Fig. 1D) and transmission electron microscopy (Fig. 3B), showed that the collagen lamellae were widely displaced, and the bacterial colonies did not demonstrate the typical arrayed structure. Instead, a mass of proliferating colonies of bacteria was observed.

**DISCUSSION**

The corneal stroma has a lamellar structure composed of approximately 200 to 250 flattened bundles of collagen fibrils. Within any given layer or lamella, the bundles run parallel to each other and extend the entire width of the cornea. However, the direction of the bundles, in alternate layers or lamellae, is at right angles to each other (orthogonal) in the posterior part of the corneal stroma and oblique to each other in the anterior part of the corneal stroma.10 This highly organized architecture of the stroma contributes to the natural transparency of the cornea. The transparency of the cornea provides a unique opportunity to directly visualize the pattern of spread of an infective process. Clinically, bacterial infection of the cornea evokes a rapid host response with inflammatory cells infiltrating the stroma and resulting in the formation of an abscess or an ulcer. However, in several clinical conditions,
notably herpetic viral disease, topical anesthetic abuse, and prolonged steroid usage, the host response is dampened, and the infective process can be visualized.\textsuperscript{1–3}

The model of bacterial infection of the cornea reported herein, is the first in vitro model of this type of human corneal infection. With this model, we were able to successfully replicate the special microbrial growth patterns seen in vivo. We were able to demonstrate this distinctive type of growth with gram-positive and gram-negative organisms, in compact nonturgid corneas. Loss of the compact structure of the cornea in the hydrated, turgid corneal buttons, prevented the formation of this pattern of growth. Although it cannot be directly concluded that the lamellar structure of the cornea is the cause of this kind of keratopathy, we have demonstrated that the compactness of the lamellar architecture and to some extent the lamellar structure itself, contributes to the formation of the clinical pattern seen in ICK. In nonturgid buttons, every needlelike branch of the arborescent pattern was confined to one interlamellar plane of the stroma. Several sections of each specimen were studied, and translamellar spread was not observed in any section. However, we did not examine serial sections, and therefore cannot confirm the absence of tranalamellar spread. Nonturgid corneal buttons do not lose their lamellar architecture, but the lamellae become separated from each other by fluid. This loss of compactness of the lamellar arrangement allows bacteria to grow as globular colonies in the wide spaces between lamellae, rather than as a branching network.

These observations support our hypothesis that it is the lamellar compact nature of the corneal architecture, rather than an inherent property of the infecting organism, that determines this pattern of growth.

This model also provides the opportunity to visualize directly in situ microbial migration and interaction with host corneal tissue. This should aid in the further understanding of the pathogenesis of corneal infections in particular and the mechanisms of bacterial adhesion and spread within tissues in general.

\textbf{References}