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An experimental model of mycobacterial infection under corneal flaps

C.B.D. Adan[1], E.H. Sato[1], L.B. Sousa[1], R.S. Oliveira[2], S.C. Leão[2] and D. Freitas[1]

Departamentos de [1]Oftalmologia and [2]Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, Escola Paulista de Medicina, São Paulo, SP, Brasil

Abstract

In order to develop a new experimental animal model of infection with *Mycobacterium chelonae* in keratomileusis, we conducted a double-blind prospective study on 24 adult male New Zealand rabbits. One eye of each rabbit was submitted to automatic lamellar keratotomy with the automatic corneal shaper under general anesthesia. Eyes were immunosuppressed by a single local injection of methyl prednisolone. Twelve animals were inoculated into the keratomileusis interface with 1 µl of 10^6 heat-inactivated bacteria (heat-inactivated inoculum controls) and 12 with 1 µl of 10^6 live bacteria. Trimethoprim drops (0.1%, w/v) were used as prophylaxis for the surgical procedure every 4 h (50 µl, qid). Animals were examined by 2 observers under a slit lamp on the 1st, 3rd, 5th, 7th, 11th, 16th, and 23rd postoperative days. Slit lamp photographs were taken to document clinical signs. Animals were sacrificed when corneal disease was detected and corneal samples were taken for microbiological analysis. Eleven of 12 experimental rabbits developed corneal disease, and *M. chelonae* could be isolated from nine rabbits. Eleven of the 12 controls receiving a heat-inactivated inoculum did not develop corneal disease. *M. chelonae* was not isolated from any of the control rabbits receiving a heat-inactivated inoculum, or from the healthy cornea of control rabbits. Corneal infection by *M. chelonae* was successfully induced in rabbits submitted to keratomileusis. To our knowledge, this is the first animal model of *M. chelonae* infection following corneal flaps for refractive surgery to be described in the literature and can be used for the analysis of therapeutic responses.

Correspondence
C.B.D. Adan
Av. Onze de Junho, 737/224
04041-052 São Paulo, SP
Brasil
Fax: +55-11-5082-4667
E-mail: gilmd@uol.com.br

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- Infectious eye diseases
- Keratitis
- Animal model
- *Mycobacterium chelonae*
- Lamellar keratotomy

Introduction

Non-tuberculous mycobacteria comprise more than 90 species and are ubiquitous microorganisms widely distributed in the environment. They are classified as rapidly growing mycobacteria, which grow in less than 7 days, and slowly growing mycobacteria, requiring culture for periods of 2-4 weeks (1).

Mycobacterium fortuitum, *M. abscessus* and *M. chelonae* are rapidly growing mycobacteria recognized as pathogenic to the eye. Turner and Stinson (2) described the first corneal infection caused by *M. fortuitum* after removal of a foreign body in 1965.

Keratitis caused by *M. chelonae* in patients undergoing laser *in situ* keratomileusis (LASIK) has only been described recently (3,4).

Infectious keratitis is a severe complication of LASIK that can potentially reduce the best final visual acuity. An incidence of infectious keratitis of 1:5000 was reported among patients submitted to LASIK, although the real incidence is unknown (5). With the growing number of refractive surgeries, an increase in the incidence of keratitis caused by non-tuberculous mycobacteria is expected.

M. chelonae is an opportunistic pathogen and in most cases non-tuberculous mycobacterial keratitis is related to exogenous inoculation of the microorganism. Since the LASIK interface confines the causative agent to replication under the suboptimal conditions of the stroma (6), it could be questioned whether keratomileusis itself would favor this infection.

Most patients with this infection have a predisposing injury (including surgery) and there is frequently a latent period of 2-8 weeks after corneal insult before keratitis appears (7). A clinical presentation of infectious crystalline keratopathy with an indolent, slowly progressive course and with gray-whitish, stellate, round or needle-like opacities can also be observed (6). Severe necrosis may sometimes be found (2).

Treatment of infectious keratitis may be difficult and response to therapy is slow due to delayed diagnosis, inadequate drug penetration and resistance to most conventional antibiotics (3,7,8).

We describe a new experimental model of *M. chelonae* keratitis in the eyes of rabbits undergoing automatic lamellar keratotomy. This model can be used for studies on mycobacterial keratitis, as well as for the analysis of therapeutic responses.

Material and Methods

Surgery, inoculations, clinical evaluation, and cultures were performed in a prospective and double-blind fashion.

Animals

Three experiments, each involving eight adult male New Zealand white rabbits each weighing 3.5 to 4.0 kg, were performed. Rabbits were maintained according to the Institutional Guidelines and the Statement for the Use of Animals in Ophthalmologic and Vision Research. Rabbits underwent previous biomicroscopy examination.

Bacteria

M. chelonae was isolated from a patient with a diagnosis of mycobacterial keratitis following LASIK. Bacteria were initially isolated in blood-agar, Sabouraud, and thioglycolate media, and were also cultivated in Middlebrook 7H9 broth (Difco) supplemented with oleic acid-albumin-dextrose-catalase (7H9-OADC; Becton Dickinson) and Middlebrook 7H10 agar (Difco) plates supplemented with OADC, and Luria-Bertani (LB) agar (1.5% (w/v) agar, 1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1.0% (w/v) NaCl). Identification was performed using PCR-restriction enzyme analysis, by analysis of the digestion patterns of a fragment of the *hsp65* gene, amplified by PCR and restricted with enzymes *Bst*EII and *Hae*III (9). Bacteria were kept in aliquots in 7H9-OADC plus 15% glycerol at -70ºC.

Bacteria in the initial log phase (4 days of culture in 7H9-OADC at 30°C in a shaking incubator) were prepared for inoculation. Turbidity of the bacterial suspension was visually compared to tube 5 of McFarland's scale (1.5 x 10^9 bacteria/ml) and absorbance at 600 nm was determined with a spectrophotometer (Hitachi® - U2000). One aliquot of each suspension was plated onto LB agar immediately after inoculation of the rabbits and the plates were incubated at 30°C. The bacterial concentration of the inoculum was

determined on the basis of the number of colony forming units. A control inoculum was prepared in the same way, but the bacteria were heat-inactivated for 20 min at 80°C. After inoculation of the heat-inactivated control, 1 µl of the suspension was plated onto LB agar to confirm bacterial inactivity.

Surgical procedure

Animals were anesthetized by intramuscular injections of xylazine (5 mg/kg) and ketamine (25 mg/kg). The left eye also received topical anesthesia with 0.5% proparacaine hydrochloride. Local antisepsis was performed with 10% povidone-iodine (on the fur) and 5% (on the eyes). Fur and lashes were isolated with a sterile plastic sheet. The eye was proptosed and rinsed with balanced saline solution. A line was traced on the corneal surface using a gentian violet pencil in order to aid the repositioning of the keratomileusis flap. A nasal-based corneal flap with a diameter of 9.5 mm and a thickness of 160 µm was created using an automated microkeratome - Automatic Corneal Shaper (ACS No. 196, Chiron-Bausch & Lomb, Irvine, CA, USA). The flap was lifted using a spatula and the posterior face of the disk and the bed was dried with a polyvinyl acetate sponge.

Infection of the operated eyes

One microliter containing approximately 10^6 colony forming units of live or heat-inactivated *M. chelonae* was placed into the keratomileusis interface. The suspensions were randomly distributed in a blind fashion during the experiment. After disk reposition and surface drying, subconjunctival injection of 20 mg methyl prednisolone acetate was performed to promote local immunosuppression. One drop of a wide spectrum antibiotic (0.1% trimethoprim (w/v) without preservatives) was applied immediately after the surgical procedure and every 4 h during the postoperative follow-up period for antibiotic prophylaxis. Susceptibility tests confirmed that the *M. chelonae* strain used in this study was resistant to this antibiotic at this concentration.

Postoperative evaluation

Two different examiners observed the animals postoperatively by biomicroscopy on days 1, 3, 5, 7, 11, 16, and 23 post-inoculation. Photographic documentation was obtained with a Topcon Slit Lamp (SL)-7E (Tokyo, Japan).

Animals were sacrificed when clinical signs of disease were detected (corneal infiltrates, edema, ulcer). Specimens were processed for laboratory work-up on the same day.

Cornea laboratory work-up

Removal of the corneal button was performed under aseptic conditions. The eye was proptosed, and before trephination the corneal flap was lifted and the stromal bed was scraped with a disposable blade for smears and for culture analysis. The flap was repositioned and a corneal trephination was performed using a disposable trephine 12.0 mm in diameter up to 2/3 of the corneal thickness. The anterior chamber was approached with a disposable blade and entrance was completed up to its total thickness with scissors. The button was placed in 2.0 ml 7H9-OADC culture medium and immediately processed.

One animal in each experiment was chosen in a random fashion to have its contralateral cornea (without surgery and without disease) recovered to serve as negative control (healthy contralateral eye of infected rabbits).

Specimen processing

All procedures were carried out under a laminar flow, using sterile gloves. All instru-

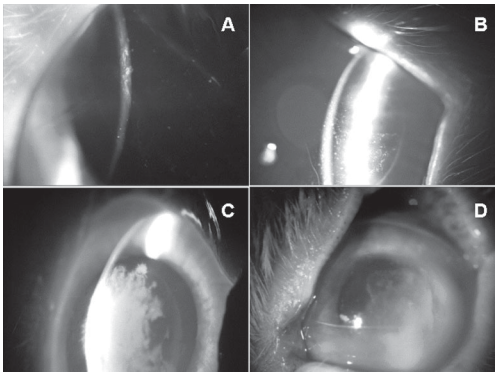

Figure 1. Clinical aspect of corneal disease by *Mycobacterium chelonae* in rabbits submitted to automatic lamellar keratotomy. *A*, Corneal interface with an initial, mild lamellar keratitis. *B*, Small and fine dots ("sands of Sahara" appearance). *C*, "Cracked windshield" appearance. *D*, Corneal abscess with feathery margins.

Table 1. Clinical and laboratory results obtained with the model of *Mycobacterium chelonae* infection of rabbits described in the present study.

	Inoculum	Corneal disease	AARB
Rabbit series I			
1	Live	+	+
2	Live	+	+
3	Live	+	–
4	Heat-inactivated	–	–
5	Heat-inactivated	–	–
6	Heat-inactivated	+	–
7	Live	+	+
8	Heat-inactivated	–	–
9	Healthy cornea control	–	–
Rabbit series II			
1	Heat-inactivated	–	–
2	Live	+	+
3	Heat-inactivated	–	–
4	Live	+	+
5	Live	+	+
6	Live	+	–
7	Heat-inactivated	–	–
8	Heat-inactivated	–	–
9	Healthy cornea control	–	–
Rabbit series III			
1	Live	+	+
2	Heat-inactivated	–	–
3	Heat-inactivated	–	–
4	Live	+	+
5	Live	Rabbit death	Rabbit death
6	Live	+	+
7	Heat-inactivated	–	–
8	Heat-inactivated	–	–
9	Healthy cornea control	–	–

AARB = alcohol-acid-resistant bacilli, identified by PCR-restriction enzyme analysis as *M. chelonae*; CFU = colony forming units. Series I, inoculum = 1.28 x 10^6 CFU; series II, inoculum = 1.06 x 10^6 CFU; series III, inoculum = 1.03 x 10^6 CFU.

ments were changed between samples in order to avoid cross-contamination. Corneal buttons were cut up into small pieces, placed in a porcelain container and then macerated in 2.0 ml 7H9-OADC. One aliquot of the suspension was diluted 1:10 in 7H9-OADC. Non-diluted and diluted aliquots were then plated onto LB agar containing antimicrobial agents to avoid secondary contamination (vancomycin hydrochloride (500 mg, 6 µg/ml; Eurofarma, São Paulo, SP, Brazil), polymixin B sulfate (5000 IU, 15 µg/ml; Sigma-Aldrich Co.), and amphotericin B (Fungizone®, 50 mg, 5 µg/ml; Bristol-Myers Squibb) and incubated at 30ºC until the appearance of colonies. Non-diluted and diluted aliquots were maintained in 7H9-OADC. The presence of mycobacteria was confirmed by Ziehl-Neelsen staining and PCR-restriction enzyme analysis.

Results

The initial biomicroscopic corneal aspect was that of a mild lamellar keratitis (Figure 1A). Clinical findings evolved to the appearance of small and fine dots ("sands of Sahara" aspect) (Figure 1B) or of a "cracked windshield" lesion (Figure 1C) without surrounding edema. A corneal abscess with feathery margins was also observed under a slit lamp (Figure 1D). Disease started on the 5th postoperative day. Eleven experimental rabbits developed clinical corneal disease and one died before developing corneal disease. Eleven control rabbits receiving heat-inactivated inoculum did not develop corneal disease. One rabbit in this group had a secondary infection with Gram-positive cocci and fungi. No healthy cornea control rabbit developed clinical disease. *M. chelonae* was isolated from 9 of 11 experimental rabbits, but not from any of the heat-inactivated inoculum control rabbits nor from any healthy cornea control rabbits.

Table 1 shows the clinical and laboratory results obtained in three series of experiments.

Discussion

LASIK is accepted as an effective surgical procedure for the correction of refractive errors (10). The rapid recovery of vision and predictability are advantages of this technique. However, complications such as infectious keratitis, although believed to be rare, have been reported in the literature and are considered to be sight-threatening (5,11). Mycobacteria, previously regarded as unusual organisms, have been recently reported as causative agents of outbreak infections following LASIK (8,12). The occurrence of keratitis in clusters following LASIK, an elective surgery with good prognosis, may be considered to be a very severe complication.

Available data about infection with the *M. chelonae-abscessus* and *M. fortuitum* groups are confusing, since these bacteria were previously described as belonging to the same subspecies or as being biovariants (13). With the recent introduction and evolution of molecular techniques, there is strong evidence that these are, in fact, two separate species (14). However, when analyzing previous studies, it is difficult to recognize and establish which species was, in fact, responsible for the disease, particularly in reports written before 1990 (14).

In the past, most experimental studies involving infection by atypical mycobacteria in rabbit eyes were performed using different strains of *M. fortuitum*. This species was probably chosen because of its strong pathogenicity to man and/or its frequent involvement in disease at that time, or even due to the lack of sophisticated methods of identification to allow separation of groups into different species.

Rabbits have been frequently used as experimental animals in ophthalmology (15) because of their eye structure (similar to the human eye), cornea size, and capability of being examined by slit lamp. In addition, the rabbit cornea can be submitted to keratomileusis. There are some difficulties in producing mycobacterial keratitis in this animal model, possibly due to their natural resistance. It has been known that rabbits are in fact resistant to infection by atypical mycobacteria which are pathogenic to man (16,17). According to Kushner et al. (18), non-tuberculous mycobacteria are virulent to mice, less so to rabbits and will not infect guinea pigs. Several attempts to develop an animal model of non-tuberculous mycobacterial keratitis have been unsuccessful. In 1966, Levenson and Harrison (16) reported an apparent high degree of resistance of the rabbit cornea to infection by *M. fortuitum* isolates from their patients.

Turner (17) reported some of the causative factors for the formation of corneal ulcer by *M. fortuitum* in rabbits. Using different methods to diminish corneal resistance prior to inoculation, as well as different techniques of inoculation, he observed that it was necessary to modify host resistance or to increase the virulence of the bacilli. He reported that pretreatment with topical corticosteroids 2 weeks prior to inoculation facilitated the development of keratitis. In 1992, Paschal et al. (19) observed that a single subconjunctival injection of methylprednisolone, an intermediate-acting corticosteroid, at the time of mycobacteria inoculation was enough to cause immunosuppression and induce indolent keratitis, and that treatment with corticosteroids before inoculation was not necessary. This approach was followed in the present study.

An important aspect of this animal model is related to the inoculum. In pilot studies, it was observed that the viability of this clinical strain of *M. chelonae* was reduced 2 h after thawing (data not shown). For this reason, inoculum suspensions were prepared 1 h before inoculation from freshly cultivated bacteria.

In order to avoid waste during flap reposition, we inoculated 10^6 bacteria/µl. Turner

Table 2. Summary of published animal models of keratitis induced by non-tuberculous mycobacteria.

Bacteria	Number of microorganisms in the inoculum	State of the organisms	Animal inoculation	Reference
M. fortuitum	6×10^4	Viable	Intrastromal	Turner (17)
M. marinum	6×10^4	Viable	Intrastromal	Turner (17)
M. fortuitum	<2000; $>10^6$	Viable or killed	Anterior chamber	Kirber et al. (20)
M. fortuitum	10^4	Viable	Intrastromal	Paschal et al. (19)
M. fortuitum	10^4	Viable	Intrastromal	Helm et al. (21)
M. fortuitum	10^4	Viable	Intrastromal	Lin et al. (22)
M. chelonae	10^4	Viable	Intrastromal	Lin et al. (22)
M. fortuitum	10^4	Viable	Intrastromal	Hu and Wang (23)
M. chelonae	10^4	Viable	Intrastromal	Hu and Wang (23)

Eyes were immunosuppressed in Refs. 17, 19, 21, 22, and 23. Ref. 17 used 0.1 ml of a 6×10^5 organisms/ml suspension. Refs. 19, 21, 22, and 23 used 10 µl of a 10^6/ml suspension.

(17), using 0.1 ml of a 600,000 organisms/ml suspension (by McFarland's technique), produced infection in rabbit corneas by stromal injection. Kirber et al. (20) injected different preparations of live and killed *M. fortuitum* into the anterior chamber of the rabbit eye and observed that preparations containing up to 2000 viable organisms were ineffective in eliciting a reaction. Animals that received an inoculum containing more than 10^7 organisms developed keratitis and reaction in the anterior chamber within 24 h. In his experimental animal model using *M. fortuitum*, Paschal et al. (19), followed by Helm et al. (21), Lin et al. (22), and Hu and Wang (23) (the last two groups using the same model and also *M. chelonae*) inoculated 10^4 organisms (10 µl, 10^6 organisms/ml) by intrastromal corneal injection to produce non-tuberculous mycobacterial keratitis. Paschal et al. (19), in 1992, confirmed the observations reported by Turner (17), showing that isolated inoculation of *M. fortuitum* into the rabbit cornea produced a self-limited stromal keratitis (17,19); see Table 2 for a summary of published animal models.

Since trauma and surgery seem to be factors predisposing to *M. chelonae* infection (24), in the present study we investigated the possibility that during LASIK there is mechanical trauma and inoculation of bacteria into the interface may occur. In previous reports, inoculation was carried out by intrastromal injection with a Hamilton syringe, but keratomileusis was not simulated.

The clinical appearance of the keratitis observed in this animal model was similar to that described in human disease (7,8): well demarcated or irregular edges with a snowflake or "cracked windshield" appearance, along with multiple, fine, small opacities in some animals. However, epithelial defects, described by Broadway et al. (7) as being universal, were not observed in this study, or in studies by other authors (8).

In conclusion, although infectious keratitis after LASIK is rare, *M. chelonae* is the most frequently reported species among the non-tuberculous mycobacteria involved in keratitis. It is a severe condition, in which diagnosis may be delayed and treatment is difficult.

We describe here an experimental animal model with great potential of producing infectious keratitis by *M. chelonae* following keratomileusis. Regardless of some differences between disease in the rabbit model and in humans, this model can be used in studies on the natural course and treatment of keratitis, in order to obtain a better prognosis for this severe and sight-threatening condition.

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