Latrunculins' Effects on Intraocular Pressure, Aqueous Humor Flow, and Corneal Endothelium

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Purpose. To determine the effects of latrunculin (LAT)-A or -B on intraocular pressure (IOP), aqueous humor flow (AHF), anterior chamber (AC) protein concentration ($[protein]_{AC}$), corneal endothelial permeability and morphology, and corneal thickness in living cynomolgus monkeys.

Methods. Topical LAT-A or LAT-B was administered to one eye, and vehicle to the other. IOP was measured by Goldmann tonometry, AHF and corneal endothelium transfer coefficient (k_a) by fluorophotometry, $[protein]_{AC}$ by Lowry assay, corneal endothelial cell morphology by specular microphotography, and corneal thickness by ultrasound pachymetry.

Results. LAT-A began to lower IOP at 6 hours and maximally reduced IOP by 4.6 mm Hg at 9 hours. LAT-B lowered IOP within 1 hour and maximally reduced IOP by 3.1 mm Hg at 6 hours. LAT-A increased AHF by 87% for 3 hours and increased k_a by 94% over 6 hours; LAT-B increased k_a by 39% over 6 hours without affecting AHF. LAT-A increased IV fluorescein entry into the cornea approximately 10 fold, but did not affect IV fluorescein entry into the AC. LAT-A increased $[protein]_{AC}$ by 25% at 2 hours but not 5.5 hours. LAT-B variably and insignificantly increased $[protein]_{AC}$ at 1 hour but not at 6.5 hours. LAT-A induced extensive corneal endothelial pseudoguttata within 1 hour, with normal cell counts by 7 days. LAT-B increased central corneal thickness maximally by 47 μm at 3.5 hours.

Conclusions. LAT-A and -B significantly reduced IOP and were consistent in their facility-increasing effect, indicating that pharmacologic disorganization of the actin cytoskeleton in the trabecular meshwork by latrunculins may be a useful antiglaucoma strategy. However, effects on corneal endothelium or ciliary epithelium are a potential safety issue. (*Invest Ophthalmol Vis Sci.* 2000;41: 1749–1758)

Latrunculins, macrolides isolated from the marine sponge *Latrunculia magnifica*, are specific, and potent actin-disrupting agents that sequester monomeric G-actin, leading to the disassembly of actin filaments.[1–3] The two most common latrunculins, latrunculin (LAT)-A and -B, cause reversible dose- and incubation time–dependent destruction of actin bundles and associated proteins in several types of cultured cells, including HTM cells.[1–7] LAT-B's effect on the morphology and actin organization in cultured hamster fibroblasts requires higher concentrations than LAT-A.[4] In living monkeys, both LAT-A and LAT-B increase outflow facility by up to fourfold, probably by disrupting the actin cytoskeleton in TM cells, in turn relaxing the TM and separating cell–cell and cell–extracellular matrix adherens junctions within it.[6,8] However, LAT-B is as effective and 10 times more potent than LAT-A.[6,8] It is not clear why the relative potency of the two drugs in vivo and in vitro are so different. The only structural difference between the two compounds is in the macrolide,[4] a large apolar part of the molecule that is probably important for permeation into the cell.[4,9] Therefore, a pharmacokinetic mechanism may be involved.[8] In addition, LAT-B is slowly inactivated by an as yet unknown serum component in cell culture medium,[4] which is not present in the protein-poor aqueous humor or the protein-free AC perfusion medium.[8]

Although latrunculins increase outflow facility and may thus have potential for anti-glaucoma treatment, it is not yet clear whether they reduce introcular pressure (IOP) or affect other ocular tissues, such as the cornea and ciliary body, which is important for safety considerations. Additionally, given their contrasting potency profiles in cultured cells versus the live monkey eye,[4,6,8] it would be interesting to know whether LAT-A and LAT-B act differently on IOP or other ocular tissues, perhaps leading to identification of related agents that have a potent effect on IOP but less effect on the ciliary body or cornea. We therefore studied the effects of LAT-A and LAT-B on IOP, aqueous humor flow (AHF), anterior segment fluid barrier

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and permeability characteristics, and corneal endothelial morphology and function in living monkey eyes.

METHODS

Drugs and Chemicals

LAT-A was obtained from Yoel Kashman, Department of Organic Chemistry, Tel-Aviv University, Tel-Aviv, Israel, and stored as a 20-mM stock solution in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) at 4°C. LAT-B was obtained from Calbiochem–Novabiochem (La Jolla, CA) and stored as a 2-mM stock solution in DMSO (Sigma) at −20°C. The LAT-A and vehicle solutions were formulated as 11.25 μl of 20-mM LAT-A stock solution or DMSO and 33.75 μl of Bárány's mock aqueous humor[10] to give a 5-mM LAT-A and 25% DMSO (vehicle) solution; 2 × 5 μl (21 μg) or 4 × 5 μl (42 μg) drops of the LAT-A solution are submaximal and maximal facility-effective doses, respectively.[6] The LAT-B and vehicle solutions for topical application (500 μM LAT-B, 25% DMSO) were formulated respectively as 11.25 μl of 2-mM LAT-B stock solution or DMSO + 33.75 μl Bárány's solution; 4 × 5 μl (approximately 4.0 μg) of the LAT-B solution is a maximal facility-effective dose.[8] All chemicals for the Lowry assay were obtained from Sigma. Fluorescein Na for intravenous (IV) injection was Fluorescite 10% (Alcon, Fort Worth, TX).

Animals and Anesthesia

Adult cynomolgus monkeys (*Macaca fascicularis*) of both sexes, weighing 2.0 to 5.5 kg, were studied. Anesthesia was induced by intramuscular (IM) ketamine (10 mg/kg) and maintained with supplemental injections of ketamine as required (5 mg/kg every 30 to 45 minutes). Between ketamine injections during a given experiment, monkeys were maintained in transfer cages after each measurement and usually were waking up. The duration of each experiment was 9 hours or less. Some animals received an extra single IOP measurement at 24 hours after a 9-hour experiment; they were allowed to fully recover from the anesthesia in their regular cages in the Animal Care Unit between the 9- and 24-hour measurements. Those monkeys used in the specular microscopy protocol also received IM acepromazine (1 mg/kg). All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with National Institutes of Health and University of Wisconsin guidelines.

Slit Lamp Biomicroscopy

A trained ophthalmologist examined all eyes by slit lamp for integrity of the corneal epithelium and endothelium, presence of flare or cells in the AC, and clarity of the lens. All animals were free of ocular abnormalities when studied.

Intraocular Pressure

IOP was determined with a minified Goldmann applanation tonometer,[11] using a cream–milk combination (Half and Half; Borden, Columbus, OH) as the tear film indicator,[12] with the monkey lying prone in a head holder and the eyes positioned 4 to 8 cm above the heart. All monkeys were examined by slit lamp before the first IOP measurement in each protocol. For each eye, two or three IOP measurements were averaged as a baseline.

After baseline IOP was measured, 21 or 42 μg LAT-A or 4 μg LAT-B was administered to the central cornea of supine monkeys in one eye and the vehicle to the opposite eye. Blinking was prevented with lid speculums during and for 5 minutes after drug administration (taking care to avoid touching the globe), to maximize drug penetration into the AC and minimize systemic absorption. After drug administration, the speculums were gently removed, and the monkeys were kept supine for another 15 minutes to further facilitate penetration of drug–vehicle solution into the AC. For LAT-A, IOP was then measured every hour for 7 to 9 hours, beginning 1, 6, or 15 hours after the 21-μg dose or beginning 1 or 6 hours after the 42-μg dose, and again at 24 hours. Each protocol included two groups of monkeys. Group one (six monkeys) underwent IOP measurement from 1 to 9 hours and again at 24 hours after the drug; group two subjects (six monkeys) were measured from 6 to 13 hours for both doses and again from 15 to 21 hours on a separate occasion for the 21-μg dose. For both doses, one monkey in group one was used again in group two; therefore, this subject's two readings, obtained at baseline or the period 6 to 9 hours after the drug on the two different occasions were averaged for data analysis. Thus, n for baseline and the period 6 to 9 hours after drug administration is 11 rather than 12. During intervals between the postdrug reading at 9 hours and that at 24 hours for group one or between the drug administration and the first postdrug reading at 6 hours for group two, the animals were returned to the Animal Care Unit for recovery. For LAT-B, IOP was measured every hour for 6 hours, beginning 1 hour after the 4-μg dose, and again at 24 hours. The animals were returned to the Animal Care Unit for recovery between the measurements at 6 and 24 hours. Anterior segments were examined by slit lamp at 3, 6, 10, or 24 hours after LAT-A, or at 1, 3, 6, or 24 hours after LAT-B.

Aqueous Humor Flow and Corneal Endothelial Permeability

AHF rate was determined noninvasively by scanning ocular fluorophotometry (Fluorotron Master; Coherent, Palo Alto, CA, and Ocumetrics, Mountain View, CA). Each monkey was examined biomicroscopically, and background fluorescence in the cornea and AC was determined before fluorescein administration. On the afternoon preceding LAT-A or B administration (usually ~4–5 PM), one drop of 0.5% proparacaine HCl (Alcaine, Alcon) was administered bilaterally (to enhance corneal penetration of fluorescein) to supine ketamine-anesthetized monkeys. Five minutes later, five 2-μl drops of 2% fluorescein Na (Alcon) were applied to the central cornea bilaterally at 30- to 60-second intervals. Blinking was prevented between drops and for 5 minutes after the final drop with lid speculums. The next morning (usually ~9–10 AM), after a saline flush of the conjunctival sac, four 5-μl drops of 5 mM LAT-A (42 μg) or four 5-μl drops of 500 μM LAT-B (4 μg) were administered to the central cornea of one eye, and four 5-μl drops of vehicle (25% DMSO) to the opposite eye, with 1-minute intervals between drops in each eye with the monkey supine. Blinking was prevented as described. Beginning 30 minutes later, corneal and AC fluorescence was measured every 30 minutes for 6 hours. AC volume was estimated from corneal thickness, AC depth, corneal curvature, and corneal diameter, all determined optically.[13] AHF rate and k_a (the

transfer coefficient for fluorescein exchange across the corneal endothelium into the AC, calculated as the area of the corneal endothelium divided by the volume of the cornea and multiplied by the permeability coefficient of endothelium from aqueous to cornea[14]) were then calculated by a modification[15] of the method of Jones and Maurice.[16] Baseline AHF was measured in a similar way without drug or placebo 2 to 13 days before and 10 to 26 days after LAT-A or -B administration.

Blood–Aqueous Barrier Permeability to Systemic Fluorescein

Topical LAT-A (42 μg) was given to one eye and vehicle to the opposite eye as described, 1 hour before intravenous injection of fluorescein Na in the saphenous vein (10 mg/kg in 500–600 μl) followed by a 4-ml saline flush. The concentration of fluorescein ([fluorescein]) in the cornea and AC was determined by fluorophotometry 15, 30, 45, 60, 90, 120, 180, and 240 minutes after fluorescein injection.

Aqueous Humor Protein

Approximately 5.5 hours after LAT-A and vehicle (25% DMSO) administration or approximately 6.5 hours after LAT-B and vehicle (25% DMSO) administration, when AHF measurements had been completed, a sample of AH was obtained under a surgical microscope (Carl Zeiss, Thornwood, NY), using a 30-gauge needle connected by polyethylene tubing to a tuberculin syringe. The needle was threaded through the corneal stroma for approximately 6 mm, then directed into the AC so that the wound was self-sealing. AH entered the tubing by very gentle suction with the tuberculin syringe; only occasionally was brief mild pressure on the cornea with a needle holder required to promote the initial flow of AH into the tubing through the very thin needle. Approximately 60 to 80 μl of AH was removed, leaving a shallow but not completely flattened AC. In separate protocols with different monkeys, AH was similarly obtained 2 hours after 42 μg LAT-A or 1 hour after 4 μg LAT-B to one eye and vehicle to the opposite eye. The AH samples were stored at −20°C for up to 24 hours. Protein concentration in each sample was assayed by the Lowry method[17,18] in duplicate and the result averaged. Duplicate sets of protein standards containing 0, 1, 3, 5, 10, 20, 40, or 60 μg bovine serum albumin were assayed by the same method and results averaged and graphed to give a linear equation that was used to estimate the protein content in the AH samples. Optical density of each sample was measured at 660 nm using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY).

Ultrasonic Pachymetry

Corneal thickness was measured using an ultrasonic pachymeter (model 1000; DGH Technology, Solana Beach, CA). All eyes were examined by biomicroscopy before baseline mea-

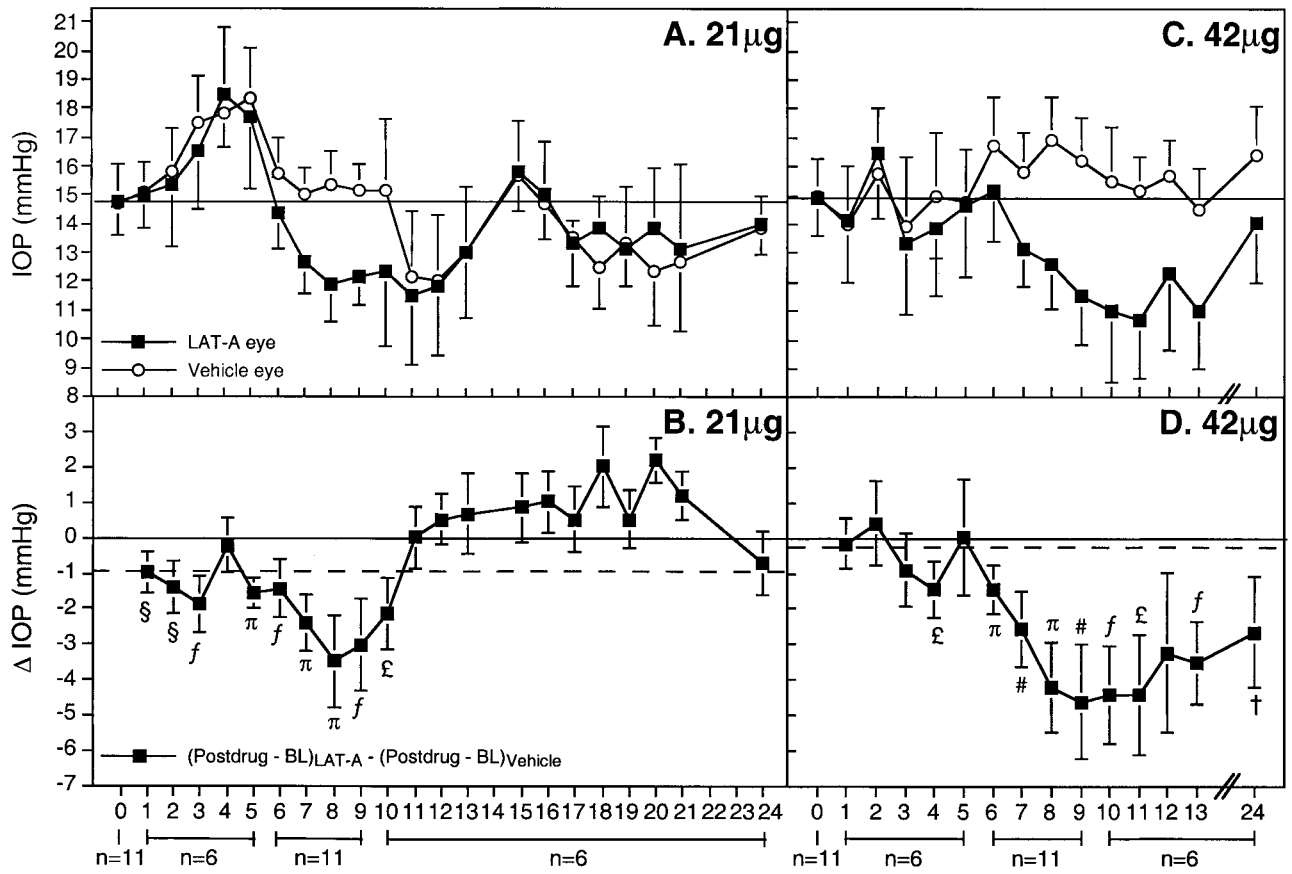

Figure 1. Effect of topical 21 μg (**A**, **B**) or 42 μg (**C**, **D**) LAT-A on IOP. *Solid line:* baseline (BL); *dashed line:* initial difference between eyes at time 0. Data are mean ± SEM for number of monkeys indicated below the abscissa, each contributing one LAT-A–treated and one vehicle-treated eye. Differences between eyes after correcting for baselines (**B**, **D**; [LAT-A-BL] − [Veh-BL]) were significantly different from 0.0 (two-tailed paired *t*-test: †$P < 0.1$, £$P < 0.05$, §$P < 0.025$, *f*$P < 0.02$, #$P < 0.01$, and π$P < 0.005$).

surements. Monkeys were placed supine in head holders. The central cornea was measured once, and the peripheral cornea was measured four times midway between the center and the limbus on the vertical and horizontal axes. For each point in each eye, two baseline thickness measurements were averaged, and 4.0 μg LAT-B or 25% DMSO was administered to opposite eyes in the LAT-B protocol, or 25% DMSO or Bárány's solution administered to opposite eyes in the DMSO control protocol. The eyelids were held open manually during and for 5 minutes after drug or vehicle administration. Corneal thickness was measured every 30 minutes for 6 hours and again at 24 hours.

Corneal Specular Microscopy

The corneal endothelium of both eyes was photographed using a specular microscope (provided by Charles J. Koester, Columbia University, New York, NY) before and 1 hour, 3 hours, 3 days, 7 days, and 14 days after topical administration of 21 or 42 μg LAT-A to one eye and vehicle to the opposite eye. Corneal endothelial cell densities were estimated by counting cells in a reference grid placed over the photograph. Cells in four separate squares were counted and averaged in each photograph. All cells completely inside the square and any cells touching two of the lines making up the square were counted. The number of cells was multiplied by 100, to compensate for the magnification of the camera and to determine cell density in cells per square millimeter. Endothelial cell morphology was evaluated subjectively by a trained corneal specialist.

Data Analysis

Data are presented as mean ± SEM for n eyes or animals as indicated. Pre- or post-LAT-A– or -LAT-B–treated eyes versus contralateral control eyes; post-LAT-A–, post-LAT-B–, or post-vehicle–treated eyes versus ipsilateral baseline; and baseline-corrected post-LAT-A– or LAT-B–treated eyes versus control eyes were compared by using a two-tailed paired t-test. Differences were compared with 0.0, and ratios were compared with 1.0.

Results

Intraocular Pressure

For each dose of LAT-A, the data were pooled so that continuous time lines could be created. The same monkeys were used for each time protocol whenever possible. The baseline IOPs for monkeys included in more than one time protocol for the same dose were averaged to provide one baseline IOP for each eye. For LAT-A, baseline IOP was approximately 15 mm Hg. The 21- μg dose of LAT-A lowered IOP 5 to 10 hours after drug administration, with the maximal decrease of 3.48 ± 1.29 mm Hg below baseline ($n = 11$, $P < 0.005$) at 8 hours (Figs. 1A, 1B). The 42- μg LAT-A dose lowered IOP 6 to 13 hours after drug administration, with maximal reduction of 4.60 ± 1.61 mm Hg below baseline ($n = 11$, $P < 0.01$) at 9 hours. IOP still had not clearly returned to baseline by 24 hours after the higher dose (Fig. 1C, D).

After LAT-B, IOP decreased from 17 mm Hg to 15 mm Hg within 1 hour and to 13.5 mm Hg at 6 hours. After adjustment for baseline and contralateral control eyes, the maximal reduction of 3.1 ± 1.2 mm Hg ($n = 8$, $P < 0.02$) occurred 6 hours after drug administration; some IOP effect may have remained at 24 hours (Fig. 2).

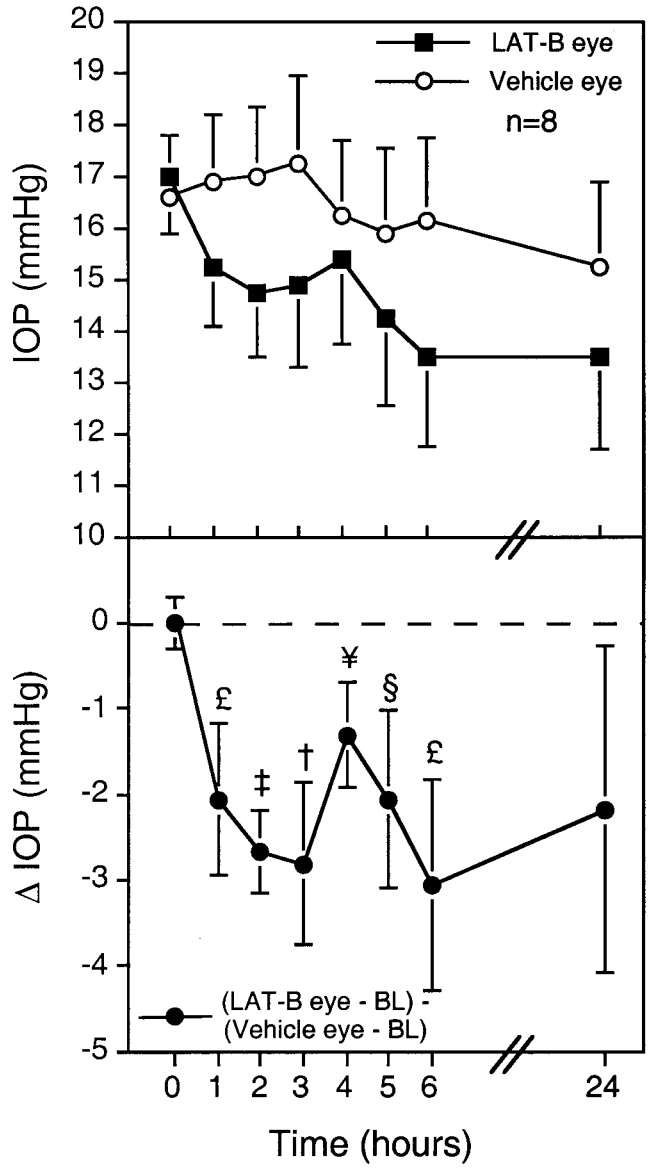

Figure 2. Effect of topical 4.0 μg LAT-B on IOP. *Dashed line* represents no change from time 0 baseline (BL). Data are mean ± SEM for eight monkeys at that time, each contributing one LAT-B–treated and one vehicle-treated eye. IOP reduction significantly different from 0.0 by the two-tailed paired t-test for differences: §$P < 0.025$, £$P < 0.02$, ¥$P < 0.01$, †$P < 0.005$, and ‡$P < 0.001$.

Slit lamp examination after LAT-A or LAT-B and vehicle (25% DMSO) administration showed mild transient corneal epithelial cloudiness in both eyes, more pronounced in the LAT-treated eyes. Four LAT-A–treated eyes and one LAT-B–treated eye showed flare ($1–2^+$ on a scale of $0–4^+$), evidence for blood–aqueous barrier breakdown but recovered by 24 to 48 hours. None of the control eyes exhibited flare. The most obvious effect of LAT-A or LAT-B was on the corneal endothelium. One to 5 hours after drug administration, the cell borders in almost all LAT-A– or LAT-B–treated eyes were indistinct, and the cells could not be counted. However, almost all drug-treated eyes exhibited innumerable small, brightly refractile,

TABLE 1. Effect of 42 μg Topical LAT-A on AHF and k_a

	Hour 0.5–3.0			Hour 3.5–6.0			Hour 0.5–6.0		
	LAT-A	Vehicle	LAT-A/Vehicle	LAT-A	Vehicle	LAT-A/Vehicle	LAT-A	Vehicle	LAT-A/Vehicle
AHF									
BL	1.97 ± 0.21	1.92 ± 0.22	1.03 ± 0.03	1.89 ± 0.13	1.97 ± 0.18	0.98 ± 0.04	1.93 ± 0.15	1.96 ± 0.19	1.00 ± 0.02
Rx	3.48 ± 0.30	1.87 ± 0.21	1.94 ± 0.14‡	1.87 ± 0.25	1.88 ± 0.18	1.00 ± 0.09	2.65 ± 0.25	1.80 ± 0.20	1.51 ± 0.08‡
Rx/BL	1.82 ± 0.14	1.00 ± 0.09	1.87 ± 0.13‡	1.00 ± 0.12	0.98 ± 0.08	1.01 ± 0.07	1.38 ± 0.09	0.92 ± 0.06	1.51 ± 0.06‡
k_a									
BL	5.45 ± 0.19	5.21 ± 0.16	1.05 ± 0.02*	5.74 ± 0.16	5.77 ± 0.21	1.00 ± 0.03	5.58 ± 0.14	5.50 ± 0.15	1.02 ± 0.02
Rx	9.96 ± 0.59	4.79 ± 0.44	2.15 ± 0.17‡	9.25 ± 1.31	5.37 ± 0.37	1.69 ± 0.13†	9.61 ± 0.51	4.93 ± 0.31	1.98 ± 0.10‡
Rx/BL	1.84 ± 0.13	0.91 ± 0.06	2.06 ± 0.16‡	1.59 ± 0.19	0.93 ± 0.06	1.68 ± 0.10‡	1.72 ± 0.08	0.89 ± 0.04	1.94 ± 0.09‡

Topical administration of vehicle or 42 μg LAT-A in contralateral eyes of eight monkeys. BL, average of baseline AHF or k_a 2 to 8 days before and 10 days after LAT-A treatment; Rx, after drug administration. AHF data are mean ± (SEM) microliters per minutes. k_a Data are mean ± (SEM) × 10^{-3}/minute. Indicated times are hours after treatment. Ratios are unitless.

* $P < 0.05$, † $P < 0.005$, ‡ $P < 0.001$ by the two-tailed paired *t*-test for ratio different from 1.0.

granule-like spots on the endothelium, presumably representing the endothelial cells. The spots could not be counted from the specular microphotographs, because they were not as clear on the photographs as in slit lamp examination. Eyes receiving the lower dose of LAT-A had fewer spots, usually localized to the central area of the endothelium at 3 hours, and recovered almost completely by 6 hours. Half of the eyes receiving the higher dose of LAT-A exhibited these spots over a large area of the endothelium within 3 hours, but most recovered by 10 hours. Eyes receiving 4 μg LAT-B exhibited as many spots as those receiving the higher dose of LAT-A at 1 hour; by 3 hours, two eyes had returned to normal, and all other eyes had improved; and by 6 hours, the corneal endothelium of almost all treated eyes had recovered. The lens always appeared normal after both drugs.

Aqueous Humor Flow and Corneal Endothelial Permeability

The two AHF baselines for individual eyes were similar and were therefore averaged. The vehicle had no effect on flow rate during any interval. LAT-A increased apparent AHF by 87% ± 13% ($n = 8$, $P < 0.001$; Table 1) in the first 3 hours, relative to vehicle-treated controls and adjusted for baseline. During the second 3 hours there were no significant differences between drug- and vehicle-treated eyes. Overall, k_a increased by 94% ± 9% ($n = 8$, $P < 0.001$), with the increase perhaps slightly greater during the first 3 hours (106% ± 16%; $P < 0.001$) than during the second 3 hours (68% ± 10%; $P < 0.001$; Table 1). The relative difference between the first and second 3-hour intervals was 27% ± 12% ($P < 0.1$). After LAT-B administration, there was only a small, insignificant increase in AHF during the first 3 hours and little effect on AHF overall (Table 2). LAT-B increased k_a by 39% ± 5% ($n = 6$, $P < 0.001$; Table 2) during the overall 6-hour measurement period, with the greatest increase during the first 3 hours (58% ± 20%, $P < 0.05$).

AC Entry of IV Fluorescein

Fifteen minutes after IV fluorescein injection (1.25 hours after topical LAT-A or vehicle), $[\text{fluorescein}]_{\text{cornea}}$ was approximately 10 times higher in LAT-A–treated eyes than in vehicle-treated eyes. The difference decreased to only approximately 1.5 times higher after 240 minutes (Figs. 3A, 3C). $[\text{Fluorescein}]_{\text{AC}}$ increased slowly in both eyes after IV injection of fluorescein, but only after 240 minutes (300 minutes after drug instillation) was $[\text{fluorescein}]_{\text{AC}}$ higher in LAT-A–treated eyes than in vehicle-treated eyes (higher by 24% ± 3%, $n = 4$, $P < 0.025$); Figs. 3B, 3D).

TABLE 2. Effect of 4.0 μg Topical LAT-B on AHF and k_a

	Hours 0.5–3.0			Hours 3.5–6.0			Hours 0.5–6.0		
	LAT-B	Vehicle	LAT-B/Vehicle	LAT-B	Vehicle	LAT-B/Vehicle	LAT-B	Vehicle	LAT-B/Vehicle
AHF									
BL	1.92 ± 0.26	1.83 ± 0.18	1.04 ± 0.05	1.87 ± 0.20	1.82 ± 0.15	1.03 ± 0.09	1.88 ± 0.22	1.87 ± 0.16	1.00 ± 0.05
Rx	2.37 ± 0.17	1.82 ± 0.17	1.33 ± 0.12†	1.57 ± 0.18	1.71 ± 0.14	0.93 ± 0.10	1.90 ± 0.16	1.67 ± 0.12	1.14 ± 0.06*
Rx/BL	1.31 ± 0.15*	1.01 ± 0.09	1.33 ± 0.18	0.84 ± 0.05‡	0.95 ± 0.07	0.91 ± 0.10	1.04 ± 0.08	0.90 ± 0.04*	1.16 ± 0.07*
k_a									
BL	5.09 ± 0.25	4.93 ± 0.20	1.03 ± 0.03	4.84 ± 0.27	5.02 ± 0.26	0.98 ± 0.07	4.88 ± 0.22	5.06 ± 0.17	0.96 ± 0.04
Rx	7.35 ± 0.67	4.83 ± 0.68	1.62 ± 0.18§	6.30 ± 0.43	5.31 ± 0.31	1.21 ± 0.11	6.48 ± 0.38	4.87 ± 0.34	1.34 ± 0.06¶
Rx/BL	1.44 ± 0.11§	0.97 ± 0.12	1.58 ± 0.20†	1.31 ± 0.06¶	1.06 ± 0.06	1.25 ± 0.08†	1.33 ± 0.04#	0.96 ± 0.06	1.39 ± 0.05#

Topical administration of vehicle or 4.0 μg LAT-B in contralateral eyes. BL, average of baseline AHF and k_a 5 to 13 days before and 14 to 26 days after LAT-B and vehicle treatment; Rx, after drug administration. Data are mean ± SEM (AHF, microliters per minute; k_a, $\times 10^{-3} \times \text{min}^{-1}$; ratios are unitless; $n = 6$); times are hours after treatment.

Ratio ≠ 1.0 by two-tailed *t*-test: * $P < 0.1$, † $P < 0.05$, ‡ $P < 0.025$, § $P < 0.02$, ‖ $P < 0.01$, ¶ $P < 0.005$, # $P < 0.001$.

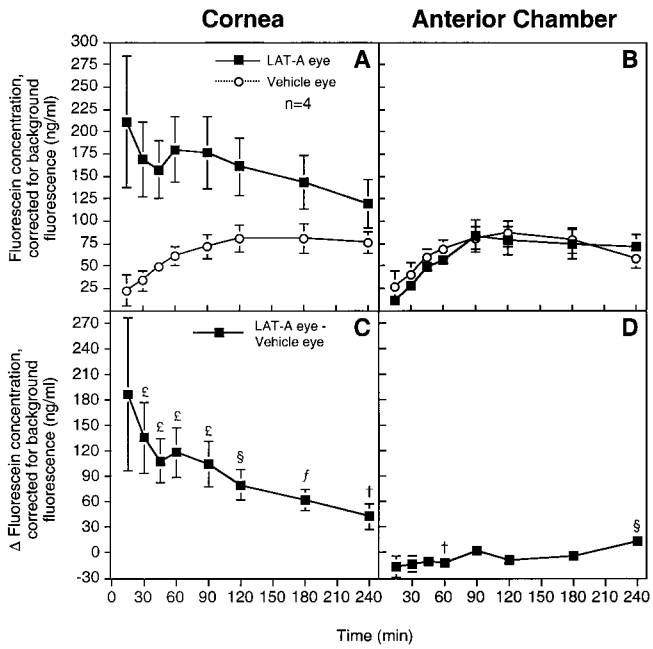

FIGURE 3. Effect of 42 μg topical LAT-A on rate of entry of fluorescein into the cornea (**A**, **C**) and the AC (**B**, **D**) after IV injection. Data are mean ± SEM for 4 monkeys, each contributing one LAT-A–treated and one vehicle-treated eye. Fluorescein was injected at time 0, 1 hour after topical LAT-A. Difference in [fluorescein] between eyes ≠ 0.0 by the two-tailed paired *t*-test for differences: †$P < 0.1$, £$P < 0.05$, §$P < 0.025$, and *f*$P < 0.02$.

Aqueous Humor Protein

Two hours after LAT-A administration $[protein]_{AC}$ in LAT-A–treated eyes was 25% ± 9% higher than that in vehicle-treated eyes ($n = 7$, $P < 0.05$; Fig. 4A). There was no difference in protein concentration at 5.5 hours (Fig. 4B). After LAT-B, $[protein]_{AC}$ was variable but was insignificantly increased overall at 1 hour and 6.5 hours (80% ± 51%; $n = 4$ and 11% ± 24%; $n = 6$ respectively; Figs. 4C, 4D) compared with contralateral control eyes.

Ultrasonic Pachymetry

Within 1 hour after LAT-B administration (Figs. 5A through 5D), the central cornea began to swell. Thickness increased to a maximum of 47 ± 17 μm greater than contralateral control eyes by 3.5 hours ($P < 0.025$, $n = 8$). The corneas then thinned over time, but were still thicker than control corneas at 6 hours (Δ = 26 ± 8 μm [6% ± 2%], $P < 0.02$). Mid-peripheral corneal thickness also increased 2 to 5 hours after LAT-B administration, with the greatest increase at 3 hours (Δ = 32 ± 8 μm [6% ± 1%], $P < 0.005$). In eyes treated with DMSO vehicle, corneal thickness increased from baseline during the first 30 minutes (Δ = 28 ± 9.6 μm [6% ± 2%], $P < 0.025$), then gradually decreased to baseline by 24 hours. By 24 hours, the central and peripheral corneal thickness in both eyes had returned to baseline. In the DMSO versus Bárány's protocol (Figs. 5E through 5H), there was a variable statistically insignificant tendency for 25% DMSO to thicken the central cornea initially (Δ = 14 ± 8.4 μm [4% ± 3%], $P < 0.2$), followed by slight but variably significant thinning of both the central and peripheral cornea (Δ ≈ 5–12 μm [~1–3%]) beyond 2 hours, compared with the contralateral control eyes receiving Bárány's solution.

Corneal Specular Microscopy

Baseline specular microphotographs showed normal corneal endothelium with cell densities between 2300 and 2950 cells/mm^2 (Fig. 6; Table 3). A few vehicle-treated eyes exhibited pseudoguttata and bright spots, especially at the higher dose of DMSO (data not shown). Cells in eyes receiving either dose of LAT-A could not be counted at 1 or 3 hours (Table 3), because the cell borders were not visible, and a few pseudoguttata and bright spots were present (Fig. 6). By day 3, cell borders in all eyes recovered, and cells became countable. The cell densities in eyes that had received 21 μg LAT-A were essentially the same as at baseline, whereas the eyes that had received 42 μg LAT-A had slightly (but not significantly) lower cell densities than baseline or contralateral eyes (Table 3). By day 7 after either dose, cell density and morphology had returned to baseline.

DISCUSSION

Previous studies have shown that LAT-A and LAT-B dramatically increase outflow facility in living monkey eyes, with LAT-B producing a stronger initial facility elevation than LAT-A.[6,8] In this study, both LAT-A and -B significantly reduced IOP in living monkeys, consistent with their facility effects. However, the IOP reduction did not occur until 6 hours after LAT-A administration; while LAT-B reduced IOP within 1 hour. This difference may be due to the smaller initial facility increase after

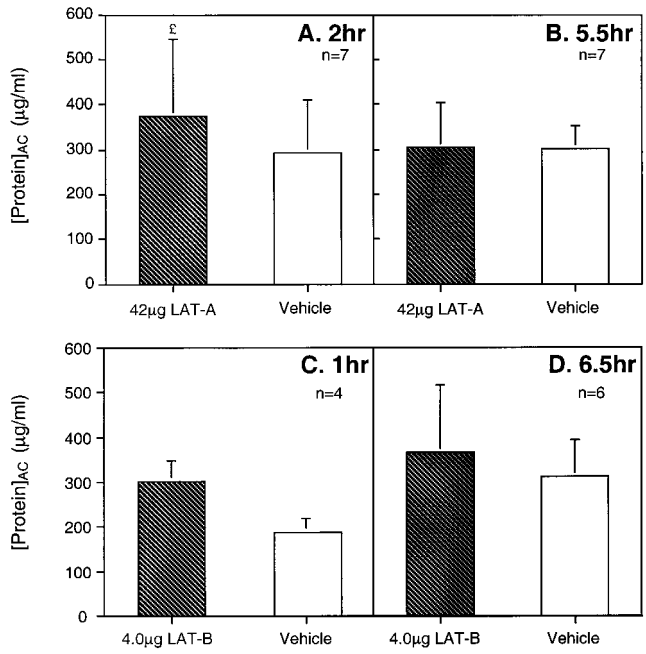

FIGURE 4. AC protein concentration (by Lowry method) 2 hours (**A**) and 5.5 hours (**B**) after application of 42 μg topical LAT-A to one eye and vehicle to the opposite eye, or 1 hour (**C**) and 6.5 hours (**D**) after applying 4.0 μg topical LAT-B to one eye and vehicle to the opposite eye. Data are mean ± SEM for *n* monkeys, each contributing one LAT-A– or LAT-B–treated eye and one vehicle-treated eye. £$P < 0.05$ for ratio different from 1.0 by the two-tailed paired *t*-test.

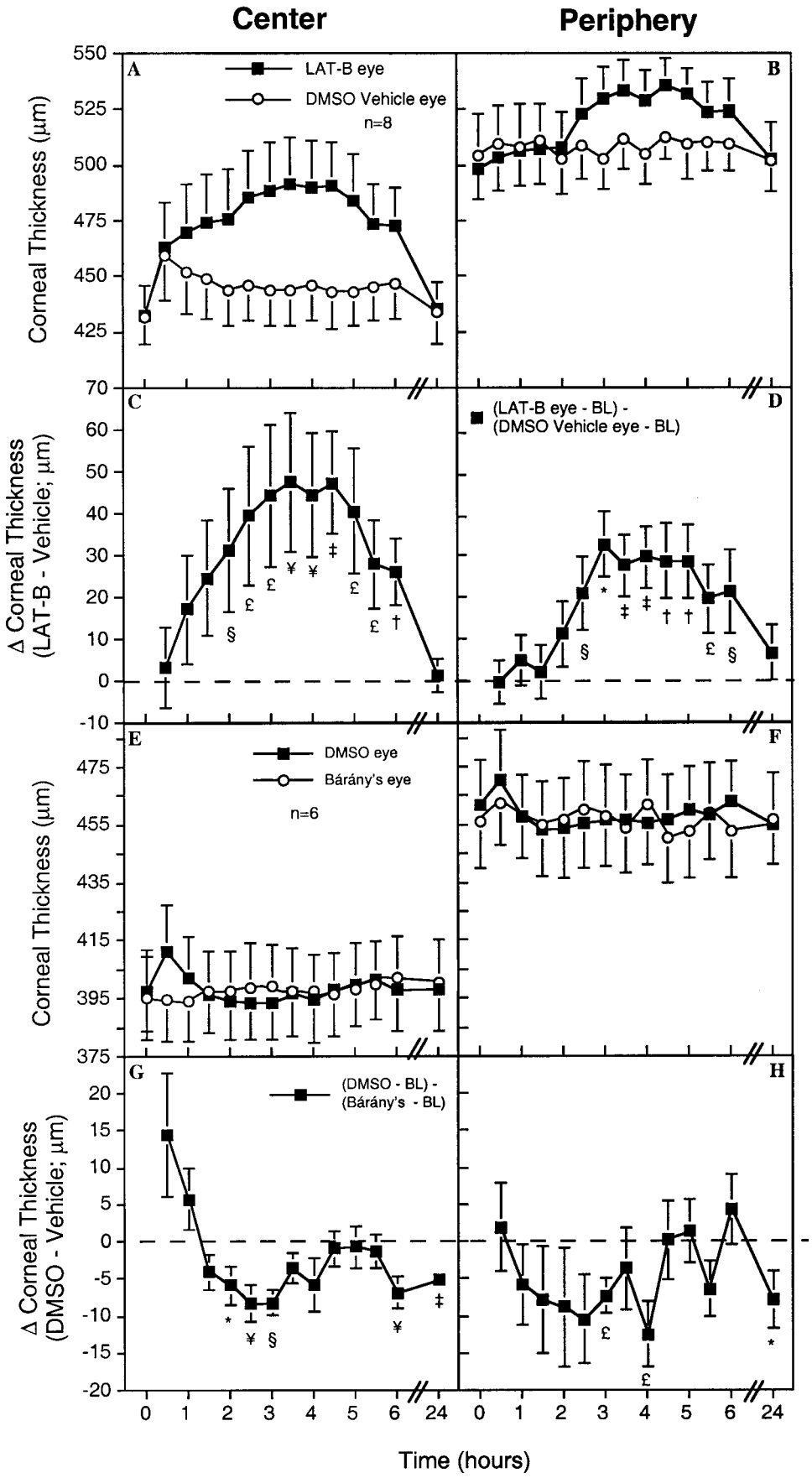

FIGURE 5. Effect of 4.0 μg topical LAT-B (**A** through **D**) or 25% DMSO (**E** through **H**) on corneal thickness as measured by ultrasonic pachymetry. *Dashed line* represents no change from time 0 baseline (BL). Data are mean ± SEM for eight (**A** through **D**) or six (**E** through **H**) monkeys, each contributing one LAT-B–treated eye and one 25% DMSO vehicle-treated eye (**A** through **D**), or one DMSO-treated and one Bárány vehicle-treated eye (**E** through **H**). Differences between eyes ≠ 0.0 by the two-tailed paired *t*-test: $^{*}P < 0.1$, £$P < 0.05$, ¥$P < 0.025$, †$P < 0.02$, ‡$P < 0.01$, and §$P < 0.005$.

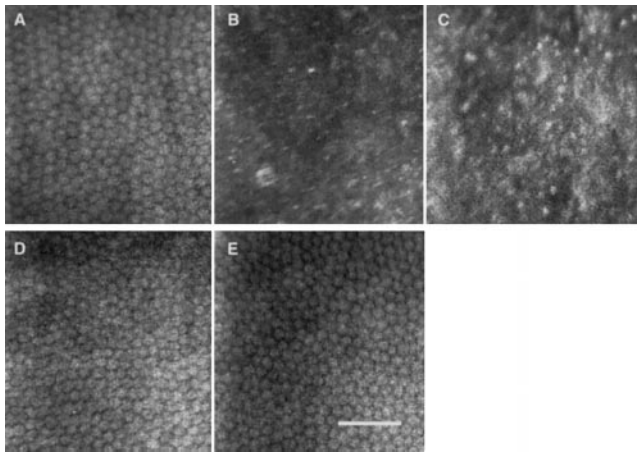

FIGURE 6. Monkey corneal endothelial cells before (**A**; baseline) and 1 hour (**B**), 3 hours (**C**), 3 days (**D**), and 7 days (**E**) after application of 42 μg topical LAT-A, by specular microscopy. Bar, 1 μm.

LAT-A, compared with LAT-B,[6,8] or to LAT-A, but not LAT-B, initially increasing AHF (described later). Other unknown mechanisms also may be involved.

In normally hydrated human corneas, IOP measured by applanation tonometry increases with the corneal thickness.[19,20] However, applanation measurements of IOP in edematous corneas may be artifactitiously low, due to the increased sponginess of the edematous cornea. In essence, the epithelium and stroma are being applanated, rather than the entire cornea, and the measurement reflects intracorneal rather than just IOP.[21] In this study, LAT-B thickened the central cornea of the live monkey eye, and presumably LAT-A would do the same. However, the LAT-A– or LAT-B–induced IOP reduction is probably not related to changes in corneal thickness, because (1) LAT-B only transiently and slightly thickened the central cornea, with the increase being approximately 47 μm at 3.5 hours (maximal change; only approximately 10% thicker compared with normal monkey cornea) or approximately 26 μm at 6 hours (only approximately 6% thicker than normal); (2) the maximal IOP reduction after LAT-B occurred at 6 hours rather than at 3.5 hours.

LAT-A increased our estimate of AHF by 87% during the first 3 hours after its application. However, what we calculated as increased AHF could also reflect changes in the cornea and blood–aqueous barrier that increase apparent fluorescein clearance without a true change in flow rate. Maus and Brubaker[22] recently demonstrated an apparent increase in flow rate of 132%, without a simultaneous increase in IOP, after dilating the pupil with tropicamide and phenylephrine. They concluded that an actual increase in flow of that magnitude was unlikely and that fluorescein may have left the AC through the dilated pupil as well as through conventional outflow.

When we determine AHF rate from the clearance of fluorescein, we assume that the AC and cornea behave as a two-compartment system and that most of the fluorescein leaves by outflow, whereas a fixed amount (representing approximately 10% of normal daytime flow) leaves by diffusion. We also assume that the fluorescence we measure accurately represents the mean concentration of fluorescein in the cornea and AC. Drugs, such as LAT-A, that change structural properties of the anterior segment could violate these assumptions by altering the route and rate of fluorescein clearance as well as our ability to measure it accurately. Thus, we cannot determine whether the apparent increase in flow measured shortly after LAT-A administration represents a true increase in AHF or an overestimate of flow rate, because of limitations in our model of the anterior segment and in our ability to measure fluorescein in this changing environment.

If this system behaves as a two-compartment model, $[\text{fluorescein}]_{\text{cornea}}$ could increase only after fluorescein passed through the AC after IV injection of fluorescein. However, $[\text{fluorescein}]_{\text{AC}}$ did not increase, while $[\text{fluorescein}]_{\text{cornea}}$ increased by 10-fold. Perhaps $[\text{fluorescein}]_{\text{AC}}$ was partially hidden by quenching from the increased $[\text{protein}]_{\text{AC}}$ after LAT-A. Possible explanations for the high $[\text{fluorescein}]_{\text{cornea}}$ may be corneal swelling or edema, changes in reflectance of the corneal endothelium, or a high fluorescein level in the tears. Without additional experiments, we do not have a good expla-

TABLE 3. Effect of Topical LAT-A on Corneal Endothelial Cell Counts

	21 μg LAT-A				42 μg LAT-A			
	LAT-A	Vehicle	LAT-A/Vehicle	LAT-A-Vehicle	LAT-A	Vehicle	LAT-A/Vehicle	LAT-A-Vehicle
BL	2538 ± 75	2638 ± 139	0.97 ± 0.03	−100 ± 86	2644 ± 128	2444 ± 63	1.08 ± 0.03*	200 ± 87
Exp								
1 hour		2463 ± 74				2175 ± 363		
3 hours		2344 ± 112				2619 ± 66		
3 days	2488 ± 63	2444 ± 98	1.02 ± 0.03	44 ± 62	2188 ± 430	2469 ± 193	0.86 ± 0.11	−281 ± 248
7 days	2488 ± 97	2556 ± 106	0.98 ± 0.06	−69 ± 140	2494 ± 166	2400 ± 105	1.04 ± 0.03	94 ± 68
14 days	2694 ± 28	2694 ± 130	1.01 ± 0.06	0 ± 147	2581 ± 143	2506 ± 103	1.03 ± 0.05	75 ± 108
Exp/BL								
1 hour		0.94 ± 0.03				0.89 ± 0.14		
3 hours		0.89 ± 0.05				1.07 ± 0.03*		
3 days	0.98 ± 0.01	0.93 ± 0.03*	1.06 ± 0.02†	0.05 ± 0.10	0.83 ± 0.14	1.01 ± 0.06	0.80 ± 0.11	−0.18 ± 0.10
7 days	0.98 ± 0.03	0.98 ± 0.05	1.02 ± 0.08	0.01 ± 0.07	0.94 ± 0.04	0.98 ± 0.02	0.96 ± 0.02	−0.04 ± 0.02
14 days	1.06 ± 0.03	1.02 ± 0.01	1.04 ± 0.03	0.04 ± 0.03	0.98 ± 0.02	1.03 ± 0.02	0.95 ± 0.03	−0.05 ± 0.03

Effect of 21 or 42 μg LAT-A (2 × 5 μl or 4 × 5 μl of 5-mM solution topically) on corneal endothelial cell counts. Data are mean ± SEM cells per square millimeter for four cynomolgus monkeys, each contributing one LAT-A–treated and one vehicle-treated eye. BL, baseline cell counts; Exp, cell counts after LAT-A or vehicle administration.

Ratios different from 1.0 by the two-tailed paired *t*-test: * $P < 0.1$; † $P < 0.05$.

nation for this phenomenon. Nevertheless, the absence of a measurable increase in $[\text{fluorescein}]_{AC}$ indicates that blood–aqueous barrier breakdown is at most minimal, consistent with the small and transient increases in $[\text{protein}]_{AC}$ measured by the Lowry assay and with the slit lamp findings during IOP measurement in which 4 of 11 LAT-A– and 1 of 8 LAT-B–treated eyes exhibited flare in the AC. The AC flare lasted approximately 24 to 48 hours, far longer than the period of increased $[\text{protein}]_{AC}$ determined by Lowry assay. However, this difference does not seem contradictory, because (1) not all monkeys exhibited AC flare; (2) AC flare was found during repeated IOP measurement by applanation tonometry, which necessitated corneal contact, whereas the $[\text{protein}]_{AC}$ measurement 5.5 hours after LAT-A was taken after only noncontact fluorophotometry. Weak barrier destabilizing effects of LAT-A and repeated corneal contact tonometry could have been additive. In any event, neither the flare nor the protein increase was substantial. LAT-B seems to have even a less consistent effect on the $[\text{protein}]_{AC}$ compared with LAT-A.

Corneal endothelial cells are held together by apical and lateral junctional complexes.[23,24] The barrier function of the endothelium depends in part on the state of these junctions.[14,25] Cytochalasin B, a fungal metabolite that affects the actin microfilament system by a complex mechanism,[26,27] disrupts the apical microfilament network of corneal endothelial cells, causing a change in corneal endothelial morphology and increasing corneal thickness.[14,28,29] After topical administration of LAT-A, corneal endothelial cell borders were transiently indistinct. This could represent disruption of cell-cell junctions.[30] However, cell shape changes and swelling could simply reorient the cell borders so that they were no longer perpendicular to the incident light, thereby diminishing specular reflected light, and rendering the cell periphery and borders indistinct. The central surface of the swollen cells would still be perpendicular to incident light and would appear as a bright central reflex.[31] Biomicroscopy revealed that both LAT-A and -B transiently produced such innumerable small, brightly refractile, granulelike spots, or pseudoguttata, on the corneal endothelium. Specular microscopy after topical LAT-B is needed to clarify whether LAT-B is gentler to the cornea than LAT-A.

The morphologic changes in the corneal endothelium could in turn induce functional changes, as indicated by the increased k_a, representing increased corneal endothelial permeability. Similar to AHF, LAT-B–induced k_a enhancement was much smaller than that induced by LAT-A, although LAT-B still transiently and mildly increased cornea thickness. Nonetheless, all these morphologic and functional changes in the corneal endothelium induced by LAT-A and -B were apparently reversible, indicating that the cells were not lost.

Collectively, that both LAT-A and B increase outflow facility and reduce IOP suggests their potential as antiglaucoma medications. However, their effects on the cornea, ciliary body, and blood–aqueous barrier are potential safety issues. LAT-B induces a stronger initial facility increase,[8] earlier IOP reduction and smaller, less consistent changes in AHF, k_a, and $[\text{protein}]_{AC}$ than does LAT-A, suggesting that LAT-B may be a better choice. The reason for these differences is not clear yet but could be the different sensitivities of ocular tissues to the two drugs.

Different drug administration strategies may also reduce the side effects. It would be of interest to administer lower concentrations of LAT-A over a longer time to see whether outflow resistance and IOP decrease without affecting the cornea or ciliary body. The high concentration–small volume formulations used in our topical drug protocols to avoid systemic and contralateral effects in the small cynomolgus monkey place the cornea at a disadvantage. The clinician would use lower concentrations in larger volumes, spreading the drug more evenly over the entire corneal surface and exposing the central cornea to a much lower dose. Also, the use of other vehicles, delivery systems, and penetration routes that are less toxic to the cornea could be explored.

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